Therapeutic Drug Monitoring of HIV Treatment

Bridging Laboratory and Clinical Practice

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Therapeutic Drug Monitoring of HIV Treatment

Bridging Laboratory and Clinical Practice

een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

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PART I

GENERAL INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS / ACQUIRED IMMUNE DEFICIENCY SYNDROME

The first patients with the acquired immunodeficiency syndrome (AIDS) were identified in 1981[1], and in early 1983 a virus that caused AIDS was isolated[2]. At the end of 1984 it was proved that the new virus (called human immunodeficiency virus; HIV) belonged to the subfamily of lentitretroviruses and that it was a retrovirus.
Like any virus, HIV must use the cells of another organism -its host- to survive and reproduce. HIV is adapted to using the cells of the human immune system, the CD4 cells.
Retroviruses are unique in biology. Unlike other viruses, retroviruses carry their genetic code as RNA and they produce a unique enzyme called reverse transcriptase, which converts their RNA into DNA. Next the cellular machinery of the host is used for reproduction.

At the end of 2004, 39.4 million people were living with HIV; the majority of these people are living in Sub-Saharan Africa: 25.4 million. About 14,000 new HIV infections a day occurred in 2004[3].

TREATMENT OF HIV/AIDS AND ANTIRETROVIRAL DRUGS

Treatment with antiretroviral drugs has been proven to prolong survival in persons with AIDS and with asymptomatic HIV infection[4]. Furthermore, the use of highly active antiretroviral therapy (HAART) is associated with a decline in morbidity and mortality in HIV-infected persons[5]. HAART is an intensive therapy in which antiretroviral drugs from different groups are combined.
There are four main groups of antiretroviral drugs (Table 1). Each of these groups attacks HIV in a different way.

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

The first group of antiretroviral drugs to treat HIV has become available in 1987 with the introduction of zidovudine[6]. These drugs inhibit the enzyme reverse transcriptase, which is produced by HIV and needed to reproduce itself in cells. These drugs are taken up by target cells and phosphorylated to triphosphate metabolites by cellular enzymes to produce active drug[7].

Nonnucleoside Reverse Transcriptase inhibitors (NNRTIs)

The second group of antiretroviral drugs is the NNRTIs and started to be approved in 1996. Nevirapine was the first NNRTI approved[6]. These drugs work slightly different from the NRTIs in that they bind in a different way to the reverse transcriptase enzyme.

Protease Inhibitors (PIs)

The third type of antiretroviral drugs are PIs, of which the first (indinavir) was approved in 1995[6]. PIs, as the name says, inhibit protease, an enzyme that breaks down protein and is one of the main enzymes that HIV uses to reproduce itself.

Fusion or Entry Inhibitors

The fourth group of antiretroviral drugs is called fusion or entry inhibitors. The first fusion inhibitor is enfuvirtide, which has been approved since 2003[8]. This drug inhibits the attachment of HIV to the cell and in this way inhibits the reproduction of viral particles.

Table 1

NRTIs	NNRTIs	PIs	Fusion Inhibitors
Abacavir	Delavirdine	Amprenavir	Enfuvirtide
Didanosine	Efavirenz	Atazanavir	
Emtricitabine	Nevirapine	Indinavir	
Lamivudine		fosAmprenavir	
Tenofovir DF		Lopinavir	
Stavudine		Nelfinavir	
Zalcitabine		Ritonavir	
Zidovudine		Saquinavir	
		Tipranavir	

THERAPEUTIC DRUG MONITORING

The role of therapeutic drug monitoring (TDM) in the management of antiretroviral agents is a topic of increasing interest[9-14]. TDM may be used when a number of important criteria are met[14]. First a more direct intermediate measure of patient response is not available; second a large interindividual variability in pharmacokinetic parameters should exist; furthermore, there should be a good relationship between plasma drug concentrations and therapeutic or toxic effect and a narrow range of concentrations that are effective and well tolerated. Finally

drug assays should be available. TDM for protease inhibitors, nevirapine, and efavirenz is useful since there is a relationship between exposure and response[14], a large inter-patient variability exists, and the therapeutic ranges are known. Furthermore, pharmacokinetic parameters may be altered by the many drug interactions that have been described for antiretroviral drugs[15], hepatic dysfunction, and non-compliance; TDM may also be useful for NRTIs in these cases.

The practice of TDM is based on several disciplines, including pharmacodynamics, pharmacokinetics, and chemical analysis. Pharmacodynamics is the study of the biochemical and physiologic effects of drugs and their mechanisms of action. Pharmacokinetics is the study of the time course of a drug and its metabolites in the body after administration by any route and therefore analysis of drugs and metabolites is needed. Chemical analysis is the basic stone. Drugs and metabolites should be determined specifically and sensitively. This is particular difficult when large amounts of different drugs are used.

OBJECTIVE OF THIS THESIS

The objective of the studies in this thesis is to make a contribution to therapeutic drug monitoring of HIV treatment by bringing information from the laboratory into the clinical setting.

The second part of the thesis is devoted to the chemical analysis. *Chapter 1* describes the simultaneous determination of the protease inhibitors and nevirapine in human plasma. A bio-analytical method is a prerequisite for therapeutic drug monitoring. In *chapter 2* the stability of protease inhibitors in plasma of HIV-infected patients was studied because long-term stability may be needed for retrospective studies. *Chapter 3* describes the cross-reactivity of efavirenz and rifampin with a urine drug-screening assay.

The third part of this thesis focuses on the quality of therapeutic drug monitoring services. The studies in *chapters 4 and 5* present the results of an international quality control program, which was initiated to alert laboratories to deviating plasma levels of protease inhibitors and the nonnucleoside reverse transcriptase inhibitors efavirenz and nevirapine. Furthermore, the quality of the recommendations given by the participants of the quality control program, concerning adjustments of doses or regimen and possible interactions was described.

In part IV of this thesis pharmacokinetic studies are described. In *chapter 6* the penetration of lopinavir into seminal plasma is studied.

In chapter 7 and 8 the possible drug-drug interaction of tenofovir disoproxil fumarate with rifampin, efavirenz, and nevirapine was studied. *Chapter 9* describes the results of a single-dose nevirapine study.

Finally, in the general discussion the results of this thesis are brought in a wider perspective and recommendations for further research are presented.

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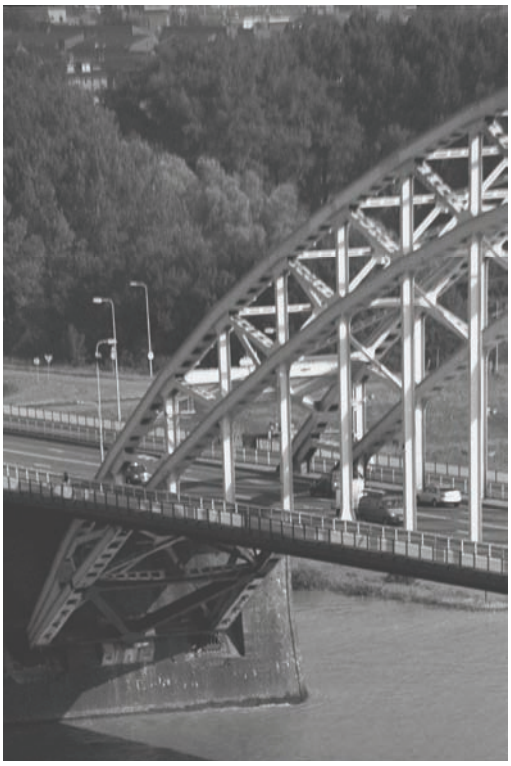

PART II

CHEMICAL ANALYSIS

CHAPTER 1

Simultaneous Determination of the HIV Drugs Indinavir, Amprenavir, Saquinavir, Ritonavir, Lopinavir, Nelfinavir, the Nelfinavir Hydroxymetabolite M8, and Nevirapine in Human Plasma by Reversed-Phase High-Performance Liquid Chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatography method for the simultaneous quantitative determination of the currently available HIV protease inhibitors amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, the active nelfinavir metabolite M8, and the nonnucleoside reverse transcriptase inhibitor nevirapine in human plasma is described. The method involved liquid-liquid extraction from plasma, followed by high-performance liquid chromatography with an OmniSpher 5 C18 column and ultraviolet detection set at a wavelength of 215 nm for the protease inhibitors and 280 nm for nevirapine. The runtime was 25 minutes. The assay has been validated over the concentration range of 0.05 to 30 mg/L for indinavir, nelfinavir, ritonavir and saquinavir, 0.07 to 30 mg/L for amprenavir and lopinavir, and 0.05 to 15 mg/L for M8 and nevirapine.

This method proved to be simple, accurate, and precise and is useful for the therapeutic drug monitoring of protease inhibitors and the nonnucleoside reverse transcriptase inhibitor nevirapine on a routine basis.

INTRODUCTION

The protease inhibitors and nonnucleoside reverse transcriptase inhibitors are potent antiretroviral drugs that have been associated with improved management of HIV infection. An important aim for treatment of HIV-infected patients is to bring the viral load in plasma to the lowest possible level, preventing disease progression. To reach this goal, therapies with different combinations of protease inhibitors, nonnucleoside reverse transcriptase inhibitors, and nucleoside reverse transcriptase inhibitors are recommended. The concentrations of the protease inhibitors and nonnucleoside reverse transcriptase inhibitors in plasma are a useful parameter for therapeutic drug monitoring, to avoid or delay viral resistance, to manage drug interactions, and to assess nonadherence.

Recently two new protease inhibitors became available: amprenavir and lopinavir.

Because of the growing number of antiretroviral drugs, many combinations of the drugs are administered to HIV-infected patients. Therefore, the availability of a method able to quantitate amprenavir, lopinavir, and nevirapine simultaneously with the other protease inhibitors would be useful.

High-performance liquid chromatography (HPLC) methods for simultaneous determination of indinavir, ritonavir, saquinavir, and nelfinavir have been reported[1-3], and the four protease inhibitors together with amprenavir[4-10] and amprenavir and nevirapine[11;12] have been described. However, no method is reported so far that describes the analysis of lopinavir. Finally, it is known that M8 is an active metabolite of nelfinavir. This article describes a validated method to analyze all protease inhibitors (M8 included) currently available and nevirapine in one analytical run based on a method previously developed in our laboratory for indinavir, nelfinavir, ritonavir, and saquinavir[1].

MATERIALS AND METHODS

Chemicals

Indinavir (IDV) was obtained from Merck (Rahway, NJ, USA); ritonavir (RTV), lopinavir (LPV), and the internal standard (IS) A860930 were obtained from Abbott (North Chicago, IL, USA); saquinavir mesylate (SQV) was obtained from Roche (Basel, Switzerland); nelfinavir (NLF) and nelfinavir hydroxymetabolite (M8) were obtained from Agouron (San Diego California, USA); amprenavir (APV) was obtained from Glaxo Wellcome (Stevenage, Hertfordshire, UK); and nevirapine was obtained from Boehringer (Mannheim, Germany). Super gradient acetonitrile and HPLC quality methanol were purchased from Labscan Analytical Sciences (Dublin, Ireland); ammonia water was purchased from OPG (Utrecht,

The Netherlands); and HPLC quality water and methyl *tert.*-butyl ether were purchased from Baker (Deventer, The Netherlands). All other reagents were obtained from Merck (Darmstadt, Germany).

Standard preparation

Stock solutions of all seven protease inhibitors and the IS were prepared in methanol and kept at 4°C. Stock solution of nevirapine was made in dimethylsulfoxide and kept at –20°C. For the preparation of the standard and quality control (QC) samples, first the stock solutions of amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, saquinavir, and nevirapine were diluted with blank plasma to concentrations that equaled the highest standard (30 mg/L for amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir and 15 mg/L for M8 and nevirapine). To achieve calibration concentrations of 0.05 to 30 mg/L, appropriate amounts of the highest standard were added to blank plasma. For the QCs, concentrations of 0.15, 1.5, and 7.5 mg/L for amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir and 0.075, 0.75, and 3.75 mg/L for M8 and nevirapine were prepared from a different stock solution.

Chromatographic equipment and conditions

The HPLC system consisted of a model P4000 solvent delivery pump, a model AS3000 autosampler, and a model UV1000 programmable wavelength UV detector. All these instruments were from Thermo Finnigan (Breda, The Netherlands). The analytical column was an OmniSpher 5 C18 column (150x4.6 mm ID; particle size, 5 µm) protected by a Chromguard RP column; both were from Varian (Middelburg, The Netherlands). Analytical runs were processed by Millennium[32] software from Waters (Etten-Leur, The Netherlands). The chromatographic separation was performed at ambient temperature with gradient elution. The mobile phase components were acetonitrile and 50 mmol/L potassium phosphate adjusted to pH 5.75 with 50 mmol/L sodium phosphate. The acetonitrile concentration was increased linearly from 36% to 61% during a 25-minute period. In 2 minutes, the acetonitrile concentration was returned to 36%. The column was then reequilibrated with the initial conditions for 3 minutes. The mobile phase flow rate was set at 1,5 mL/min. Nevirapine was detected at 280 nm, and the protease inhibitors and IS were detected at 215 nm. The injection volume was 50 µL.

Sample preparation

A 500-µL aliquot of plasma (standard, QC, patient) was spiked with IS and mixed with 500 µL 0.1 mol/L NH_4OH and 5 mL methyl *tert.*-butyl ether. After vortexing for 1 minute and centrifuging for 5 minutes, the organic layer was transferred and evaporated to dryness with

nitrogen at 37°C. The residue was dissolved in 300 µL eluent and washed with 3 mL hexane and vortexed for 5 minutes. After centrifuging for 5 minutes, 50 µL of the eluent was injected into the chromatograph. In our laboratory, 100 samples, including QC samples and calibration curve, could be extracted in 1 day. The analysis takes 2 days.

Specificity

The interference from endogenous compounds was investigated by analysis of blank plasma from six different persons who did not use any protease inhibitor or nevirapine. No interfering peaks with the peaks of the protease inhibitors, nevirapine, or IS were allowed. Possible coadministered drugs were tested at concentrations of 50 mg/L by diluting stock solutions of 1 mg/mL, 20 times.

Accuracy, precision, recovery, lower limit of quantification and lower limit of detection

Two replicates of three different concentrations of QC samples were analyzed in 40 separate runs, for determining the accuracy and precision according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines[13]. The modified EP5 protocol for 1-run/day assays was used to calculate the intraday and interday variation.

The accuracy was calculated as the average percentage of the nominal concentration. Average recovery of lopinavir, amprenavir, M8, and nevirapine was determined by comparing responses with those obtained by direct injection of the same amount of drug in mobile phase at seven different concentrations in three separate runs. For the lower limit of quantification, the percent deviation from the nominal concentration and the relative standard deviation has to be less than 20%. Samples for determining the lower limit of quantification were assayed as five replicates. The lower limit of detection was defined as the smallest quantity of drug producing a signal-to-noise ratio ≥ 3. Recovery, accuracy, and limit of quantification for indinavir, nelfinavir, ritonavir, and saquinavir were described before[1].

Stability

The stability during sample handling of lopinavir, amprenavir, and M8 was verified, subjecting samples to three freeze-thaw cycles, testing the stability in plasma at room temperature and at –20°C, and determining the stability in blood at room temperature all at different concentration levels.

Besides the stability in eluent after extraction, the stability of the standard solutions in methanol at 4°C was tested. Stability testing of nevirapine was limited to the stability in eluent. Other stability tests were performed before at our labaratory[14].

RESULTS

Chromatographic characteristics

Figure 1A to B shows chromatograms of an extracted blank plasma and a standard plasma, respectively, containing the seven protease inhibitors and nevirapine. As shown, protease inhibitors, nevirapine, and IS are well resolved. The retention times were 1.78 minutes for nevirapine, 5.25 minutes for indinavir, 8.28 minutes for amprenavir, 12.49 minutes for M8, 13.30 minutes for saquinavir, 14.34 minutes for ritonavir, 15.81 minutes for lopinavir, 17.40 minutes for IS, and 21.54 minutes for nelfinavir.

Specificity

The six blank plasma samples showed no peaks that coeluted with the protease inhibitors, nevirapine or the IS.

Of all the possible coadministered drugs tested (Table 1), delavirdine and efavirenz caused problems. Delavirdine coeluted with amprenavir. We decided that this was not a problem, because delavirdine and amprenavir are rarely coadministered in The Netherlands; elsewhere, it would be possible to change the gradient. Efavirenz had the same retention time as the IS. Therefore, there was a slight modification of the gradient. Instead of a gradient from 36% to 66 % acetonitrile described by Hugen, a gradient of 36% to 61 % was used, forcing the efavirenz to elute earlier than the IS.

When a patient used lopinavir/ritonavir, there was a metabolite of lopinavir that coeluted with ritonavir. Under the conditions described, it is not possible to measure ritonavir in the presence of lopinavir in patient plasma. This was not considered a problem, because ritonavir is only added as an inhibitor of the lopinavir metabolism.

Table 1: *Coadministered drugs for specificity*

Acetaminophen	Delavirdine	Isoniazid	Rifabutin
Acyclovir	Didanosine	Itraconazole	Rifampin
Amphotericin B	Efavirenz	Ketoconazole	Stavudine
Amoxycillin	Erythromycin	Lamivudine	Sulfamethoxazole
Atovaquone	Ethambutol	Methadone	Sulfametrol
Azithromycin	Famotidine	Oxazepam	Trimethoprim
Clarithromycin	Fluconazole	Pentamidine	Zalcitabine
Clindamycin	Folic acid	Phenytoin	Zidovudine
Caffeine	Folinic acid	Pyrazinamide	
Dapsone	Ganciclovir	Pyrimethamine	

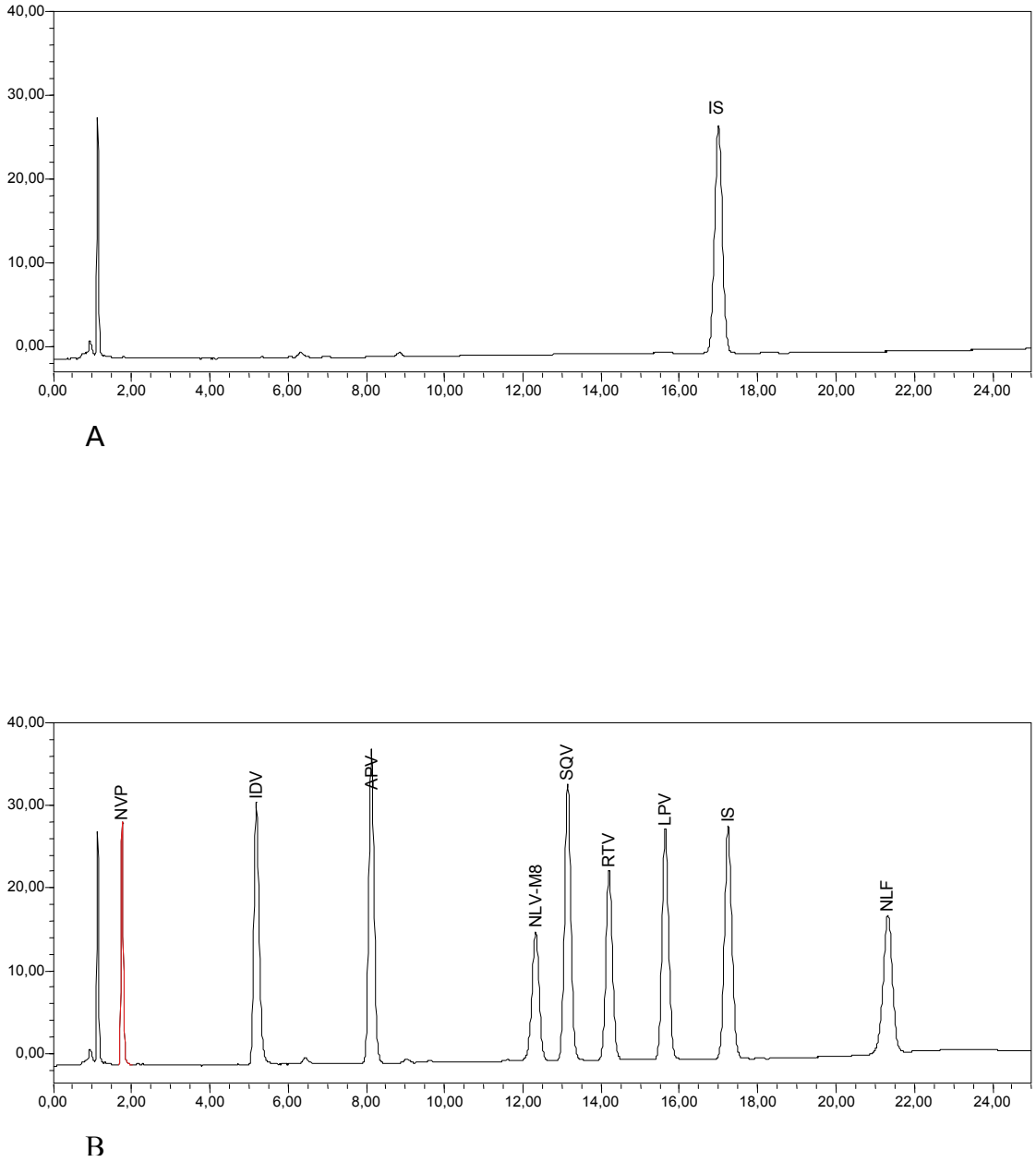

Figure 1: (A) *Chromatogram of a blank plasma sample containing 5 mg/L IS (B) Chromatogram of spiked plasma sample containing 5 mg/L IS, 3 mg/L amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir, and 1.5 mg/L nevirapine and M8*

Accuracy, precision, recovery, lower limit of quantification, and lower limit of detection

The results from the validation of the method for lopinavir, amprenavir, M8, and nevirapine in human plasma are presented in Table 2. These results show that this method is accurate (average accuracy, 97-106%) and precise (interassay coefficient of variation (CV), 2.4-8.1%; intraassay CV, 2.3-5.9%).

The extraction yield was found to be 95%, 91%, 92%, and 102% for lopinavir, amprenavir, M8, and nevirapine respectively.

The lower limit of quantification was found to be 0.07 mg/L for lopinavir and amprenavir and 0.05 mg/L for M8 and nevirapine. The detection limit was 0.006 mg/L, 0.013 mg/L, 0.014 mg/L, and 0.014 mg/L for nevirapine, amprenavir, M8, and lopinavir respectively.

The calibration curves were linear over the concentration range of 0.05 to 15 mg/L for M8 and nevirapine and 0.07 to 30 mg/L for amprenavir and lopinavir.

Table 2: *Accuracy and precision of the determination of lopinavir, amprenavir, M8, and nevirapine in plasma*

Protease inhibitor	Concentration (mg/L)	Accuracy (% CV)	Intraassay precision (% CV) (n=80)	Interassay precision (% CV) (n=80)	Recovery (mean (SD))
Lopinavir	0.487	97	5.6	4.6	95 (3.2)
	0.974	99	3.3	3.5	
	4.868	100	2.3	2.4	
Amprenavir	0.500	99	4.9	8.1	91(2.3)
	1.00	98	4.7	5.4	
	5.00	98	4.1	5.1	
M8	0.081	97	5.9	5.1	92 (0.9)
	1.49	100	3.2	3.7	
	7.08	102	2.7	3.9	
Nevirapine	0.083	101	3.8	6.2	102 (5.1)
	0.830	103	4.1	5.8	
	4.15	106	3.7	4.8	

Stability

The results of stability tests under various conditions are listed in Table 3. Under all conditions tested, lopinavir, amprenavir, and M8 proved to be stable with recoveries of at least 93.6 % of the initial concentration.

Table 3: *Stability experiments*

Protease inhibitor	Concentration range (mg/L)	Condition	Matrix	Time interval	Recovery (mean (SD))
Lopinavir	0.5-50	20°C	Plasma	8 days	103.1 (3.6)
	0.15-7.3	-20°C	Plasma	4 months	99.8 (2.2)
	0.5-5.0	freeze-thaw	Plasma	3 cycles	99.9 (3.5)
	0.3-3	20°C	Blood	5 days	101.8 (4.2)
	0.5-5.0	20°C	Eluent	4 weeks	104.7 (2.5)
	3000	20°C	Methanol	4 months	103.9 (0.8)
Amprenavir	0.5-5.0	20°C	Plasma	8 days	93.6 (4.9)
	0.16-7.5	-20°C	Plasma	4 months	101.7 (1.9)
	0.5-5.0	freeze-thaw	Plasma	3 cycles	98.7 (3.6)
	0.3-4.0	20°C	Blood	5 days	104.3 (5.5)
	0.5-5.0	20°C	Eluent	4 weeks	108.2 (1.5)
	3000	20°C	Methanol	4 months	101.0 (0.4)
M8	0.08-7.6	20°C	Plasma	8 days	100.9 (1.9)
	0.07-7.0	-20°C	Plasma	12 months	96.9 (6.0)
	0.08-7.6	freeze-thaw	Plasma	3 cycles	97.7 (0.9)
	0.4-4.0	20°C	Blood	5 days	104.4 (2.9)
	0.08-7.6	20°C	Eluent	4 weeks	104.7 (2.5)
	1500	20°C	Methanol	6 months	97.6 (0.3)
Nevirapine	0.08-4.5	20°C	Eluent	3 weeks	99.5 (3.7)

Pharmacokinetic data

The applicability of the assay for pharmacokinetic research in HIV-infected patients was demonstrated by analyzing three patient curves of amprenavir (Fig. 2A), lopinavir (Fig. 2B), and nevirapine (Fig. 2C).

The pharmacokinetic data are presented in Table 4.

According to the literature, for lopinavir the C_{min} is 5.5 mg/L, the C_{max} is 9.6 mg/L and the elimination half-life is 5 to 6 hours[15].

For amprenavir, the C_{min} is 0.33 mg/L, the C_{max} is 8.21 mg/L and the elimination half life is 8 hours[16].

For nevirapine, the C_{min} is 7.2 mg/L, the C_{max} is 4.0 mg/L and the elimination half life is 25 to 30 hours[17].

Table 4: *Pharmacokinetic data*

Patient code	A	B	C
Patient characteristics			
Gender (M/F)	F	F	M
Age (year)	35	40	40
Weight (kg)	65	67	80
Drug	Amprenavir	Lopinavir	Nevirapine
Dose (mg)	1200 BID	400 BID	200 BID
Co-medication		100 mg Ritonavir BID	
Pharmacokinetic characteristics			
C_{max} (mg/L)	7.75	8.35	5.30
T_{max} (h)	0.50	1.00	2.52
C_{min} (mg/L)	0.08	5.21	3.30
AUC_{0-12} (h.mg/L)	13.76	52.9	ND[a]
$T_{1/2}$ (h)	2.83	7.29	ND[a]

[a] Not determined

DISCUSSION AND CONCLUSION

The stability of amprenavir, lopinavir, and M8 was tested, and we found that all three were stable in plasma at –20°C; amprenavir and lopinavir were stable for at least 4 months, and M8 was stable for 12 months. After 18 months at –20°C, we found a degradation of nelfinavir and M8 of 30% and 12 %, respectively (not listed in Table 3). Turner et al[18] also reported a significant degradation of both compounds stored at –20°C for a period longer than 6 months (83% and 73 % degradation for nelfinavir and M8, respectively, after 19 months). They prepared nelfinavir and M8 calibrators in drug-free heparinized plasma for the stability testing, which cannot be completely compared with patient samples because of the addition of an organic solvent in which nelfinavir and M8 are dissolved. We used patient samples for the stability experiments.

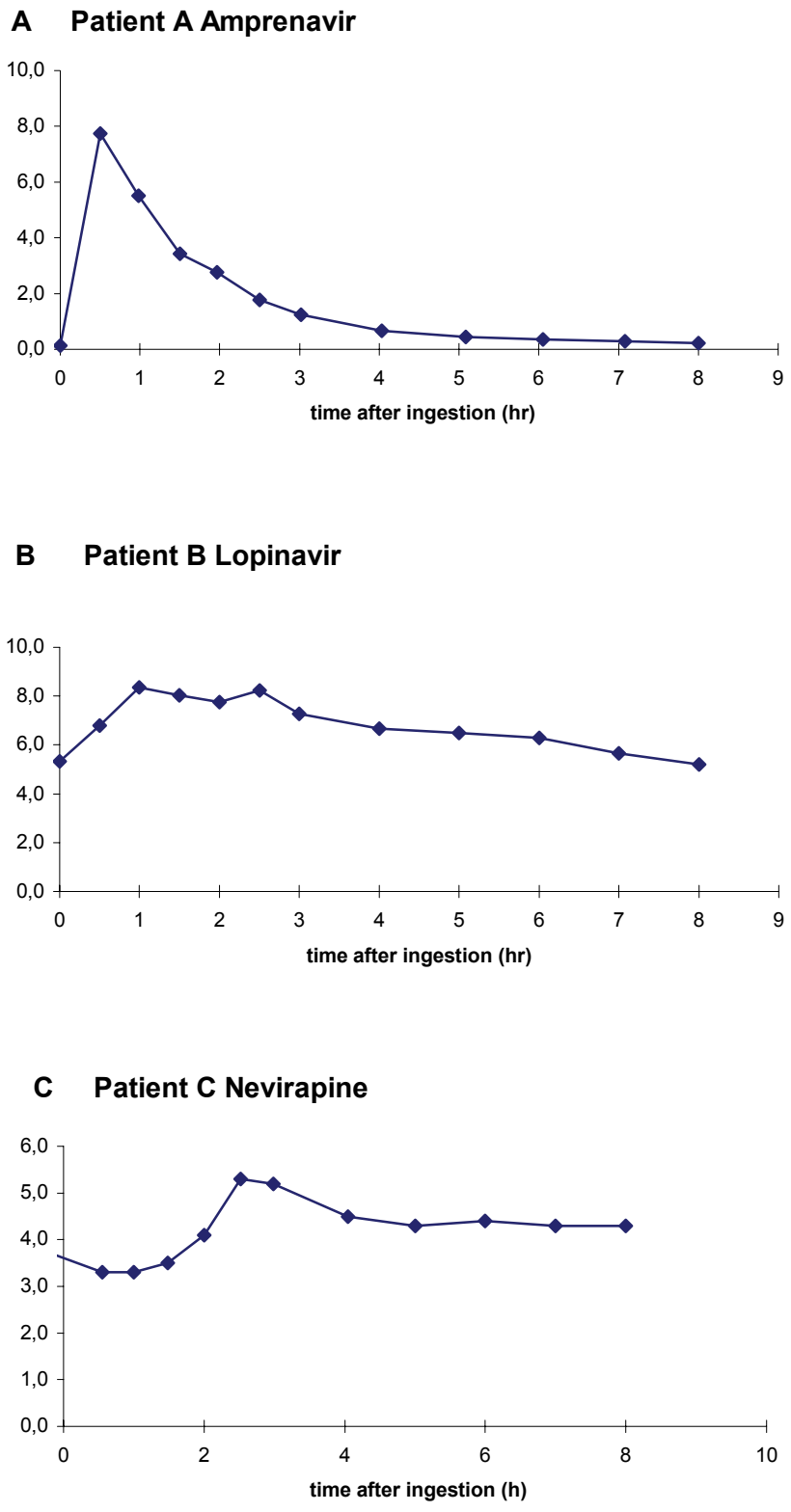

Figure 2: *Plasma concentration versus time curves for HIV-infected patients using 1200 mg amprenavir twice daily (**A**), 400 mg lopinavir twice daily (**B**), and 200 mg nevirapine twice daily (**C**).*

Analytical testing of the stability of nelfinavir has been reported before[6;8;11]. None of the authors reported degradation of nelfinavir: none of them tested longer than 6 months. Nevertheless, we think that samples must be analyzed for nelfinavir and M8 within 6 months of storage at –20°C. An alternative can be storage at –70°C[18].

Some methods that measure amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir in one run use expensive disposable cartridges for extraction[5-7;10;12].

Yamada et al[4] described an assay for five protease inhibitors but did not use an IS, and the method was not tested for interfering drugs. Villani et al[19] also did not test interfering drugs

This method measures not only amprenavir, indinavir, nelfinavir, ritonavir, saquinavir, and nevirapine in one run, but also lopinavir and M8. Efavirenz was not included in this assay, because the recovery of efavirenz after extraction was only 20 %.

In conclusion, a sensitive, specific, and validated assay for the simultaneous determination for the currently available protease inhibitors, M8, and nevirapine is described. This method can be used in a hospital laboratory for therapeutic drug monitoring of the protease inhibitors and nevirapine in patient plasma and for pharmacokinetic studies in HIV-infected patients.

The calibration curves for lopinavir (0.07-30 mg/L), amprenavir (0.07-30 mg/L), and nevirapine (0.05-15 mg/L) are appropriate for clinical drug monitoring[16;17].

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CHAPTER 2

Long-Term Stability of Protease Inhibitors in Human Plasma of HIV-Infected Patients

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ABSTRACT

Background*:* Stability of the protease inhibitors amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir in plasma at low temperatures is only studied in calibration samples. We investigated the effects of storage at -20°C and -80°C on the stability of protease inhibitors in plasma of HIV-infected patients.

Methods: Stability was determined by analyzing plasma samples stored 0 to 18 months. Plasma concentrations were compared with those at start of the study.

Results*:* Concentrations found after 18 months of storage in the freezer varied between 95% and 108% of the initial concentration.

Conclusions: Plasma samples of HIV-infected patients stored at -20°C or -80°C can still be analyzed after 18 months.

INTRODUCTION

The protease inhibitors are potent antiretroviral drugs that have been associated with an improvement in the treatment of human immunodeficiency virus (HIV) infection. The concentrations of the protease inhibitors (PIs) in plasma are useful parameters for therapeutic drug monitoring (TDM), in order to avoid or delay viral resistance, to assess toxicity, to manage drug-drug-interactions, and to assess non-adherence[1].

Numerous high-performance liquid chromatography (HPLC) methods have been published for each individual PI and for the simultaneous determination of several PIs[2]. These assays can be used for patient care and for retrospective and prospective pharmacokinetic studies.

Although many assays have been published, and analytical stability testing during assay validation is typical, long-term stability of the PIs in patient plasma has not been studied yet. There are indications that nelfinavir and its active hydroxy–*t*-butyl-amide (M8) show degradation when stored at -20°C[3]. Furthermore, Egge-Jacobsen[4] reported degradation of amprenavir at -20°C, but both these studies were performed in spiked blank plasma.

For retrospective clinical studies in particular it is of great importance to know the stability of the PIs in patient plasma in the freezer.

The aim of this study was to describe the stability of the PIs amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir in HIV-infected patient samples at –20°C and –80°C during 18 months.

MATERIAL AND METHODS

This study used HIV-infected patient samples that were collected from therapeutic drug monitoring samples during the first week of august 2003. All samples were collected in heparinized hard plastic tubes. The blood samples were centrifuged at 2,500 x g for 10 minutes. Plasma of patients using the same protease inhibitor were pooled and assayed in triplicate immediately, being the baseline value. Aliquots of the samples were stored at -20°C and -80°C. After 3, 6, 12, and 18 months samples were analyzed in triplicate to assess the concentrations and to determine the possible degradation.

Concentrations of amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir were determined by a previously described validated HPLC method with ultraviolet (UV) detection[5]. The accuracies of the method ranged from 97 % to 106% depending on the concentration level. Intraday precisions were 2.3% to 5.6% and interday precisions were 2.4% to 8.1%. The lower limit of quantification was found to be 0.04 mg/L for indinavir, M8, nelfinavir, ritonavir, and saquinavir and 0.07 mg/L for amprenavir and lopinavir.

The accuracy of the assay was externally tested by the International Program for Quality Control of Therapeutic Drug Monitoring in HIV Therapy[6;7]. During the time of this study, we participated 3 times in the quality control program, in October 2003, and March and October 2004. In this program 20% limits around the nominal concentrations are considered to be appropriate thresholds for a satisfactory measurement. Results obtained from all measurements of all three rounds of the International Quality Control Program were within these limits except for one. For amprenavir all 9 results deviated less than 17% from the nominal concentration. For indinavir all 9 results deviated less than 12% from the nominal concentration. For lopinavir and nelfinavir all 9 results deviated less than 14% from the nominal concentration. For M8 8 results deviated less than 11% and one result deviated 22% from the nominal concentration. For ritonavir and saquinavir all 9 results deviated less than 15% and 18% respectively from the nominal concentration. Results of the ongoing analysis for assessment of long-term stability should remain within 15% of their originally determined concentration according to the guidelines of the FDA for bioanalytical method validation[8].

Table 1: *Mean concentrations after storage at -20°C or -80°C (%CV)*

T	Time (m)	IDV	APV	LPV	NLF	M8	RTV	SQV
-20°C	0	2.17 (6.6)	3.17 (4.9)	9.61 (0.9)	2.29 (1.0)	0.75 (1.2)	1.32 (5.5)	0.31 (0.6)
	3	2.07 (0.9)	2.92 (2.4)	9.32 (2.7)	2.34 (1.9)	0.72 (1.1)	1.30 (0.7)	0.30 (1.0)
	6	1.93 (2.3)	2.44 (0.9)	9.67 (0.3)	2.12 (0.3)	0.62 (0.9)	1.25 (2.7)	0.29 (1.4)
	12	2.12 (1.8)	3.08 (4.3)	9.95 (2.1)	2.33 (3.4)	0.69 (3.0)	1.34 (1.7)	0.30 (2.1)
	18	2.28 (2.0)	3.01 (0.3)	10.34 (3.6)	2.31 (3.0)	0.74 (4.1)	1.41 (2.8)	0.33 (8.0)
-80°C	0	2.17 (6.6)	3.17 (4.9)	9.61 (0.9)	2.29 (1.0)	0.75 (1.2)	1.32 (5.5)	0.31 (0.6)
	3	2.05 (1.5)	3.07 (4.2)	9.57 (1.1)	2.33 (0.7)	0.73 (0.7)	1.28 (0.5)	0.30 (1.4)
	6	2.03 (0.7)	2.82 (1.0)	9.73 (1.2)	2.21 (0.4)	0.67 (2.2)	1.28 (0.9)	0.29 (2.1)
	12	2.20 (4.5)	3.21 (1.0)	10.12 (2.3)	2.47 (2.2)	0.71 (2.1)	1.36 (3.1)	0.30 (2.9)
	18	2.31 (2.4)	3.25 (2.7)	10.26 (3.3)	2.45 (3.2)	0.77 (2.1)	1.39 (2.8)	0.32 (4.5)

T= storage temperature, IDV= indinavir, APV= amprenavir, LPV= lopinavir, NLF= nelfinavir, RTV= ritonavir, SQV= saquinavir)

RESULTS

The baseline concentrations of the samples were 3.17, 2.17, 9.61, 2.29, 0.75, 1.32, and 0.31 mg/L for amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir respectively. The results are presented in Table 1. The relative concentrations (%) of amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir from samples stored at -20°C and -80°C were calculated at 3, 6, 12, and 18 months after storage with the baseline value set at 100%. The relative concentrations (%) were plotted versus the storage time for each investigated drug and at each investigated temperature (Fig. 1). The relative concentrations determined at two temperatures after 18 months were not different from the baseline values, being 106%/105%, 103%/95%, 107%/108%, 107%/101%, 102%/98%, 105%/107%, and 105%/108% for indinavir, amprenavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir respectively at -80°C/-20°C, indicating that the PIs are stable in plasma of HIV-infected patients when stored at -20°C or at -80°C for 18 months.

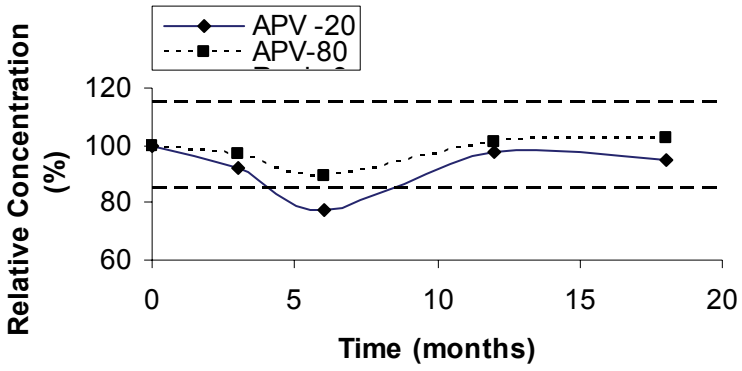
Almost all relative concentrations were found to be between the thresholds of 85% to 115 %, only the mean relative concentrations for amprenavir and M8 after 6 months of storage at -20°C were just below the 85% threshold, being 77% and 83% respectively.

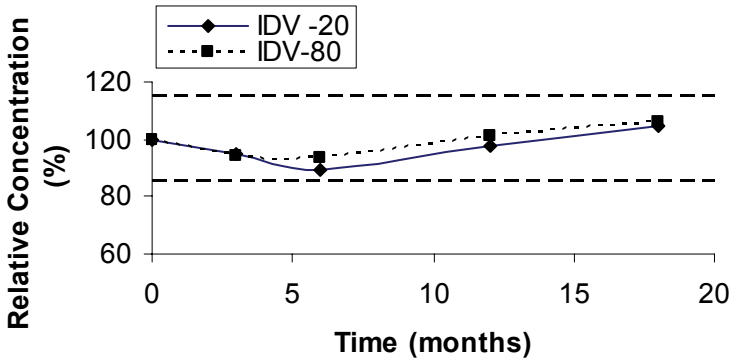
DISCUSSION

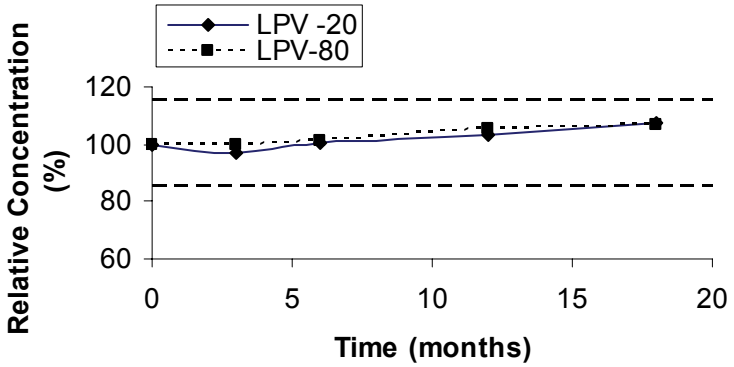
This study revealed that after 18 months of storage at either -20°C or -80°C, there is no degradation of any of the PIs amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, or saquinavir in plasma of HIV-infected patients.

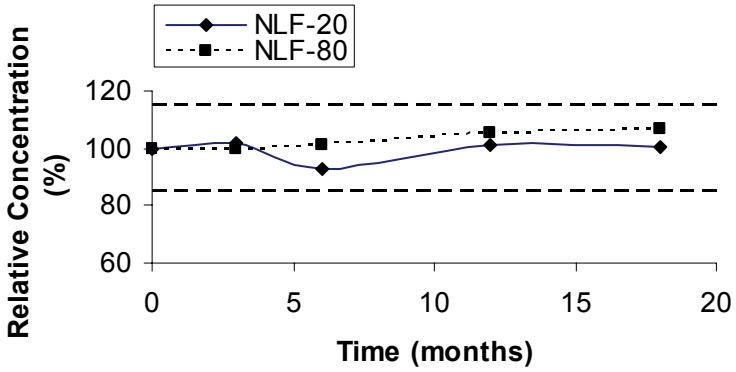
The concentrations of the PIs tested in this study were 3.17, 2.17, 9.61, 2.29, 0.75, 1.32, and 0.31 mg/L for amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir respectively, which are therapeutic concentrations for the PIs in question[9].

Although the protease inhibitors used have been available since the late nineties, the stability of these drugs in plasma of HIV-infected patients has not been studied well. In Table 2A and 2B all stability experiments during validation of HPLC assays are listed. In Table 2A the studies are listed that reported no degradation of any of the PIs. The storage time of these studies ranged from 14 days to 6 months and the samples were stored at -20°C and -70°C. In Table 2B experiments are listed that reported degradation of protease inhibitors. Only degradation of nelfinavir, M8[3;5], and amprenavir[4] was reported. All experiments listed in Table 2A and almost all experiments presented in Table 2B used spiked samples (calibration samples) for stability testing. These samples were prepared by adding PIs, which were dissolved in organic solvents, to drug-free plasma.


APV -20
APV-80
Relative Concentration (%)
120
100
80
60
0
5
10
15
20
Time (months)


IDV -20
IDV-80
Relative Concentration (%)
120
100
80
60
0
5
10
15
20
Time (months)


LPV -20
LPV-80
Relative Concentration (%)
120
100
80
60
0
5
10
15
20
Time (months)


NLF-20
NLF-80
Relative Concentration (%)
120
100
80
60
0
5
10
15
20
Time (months)

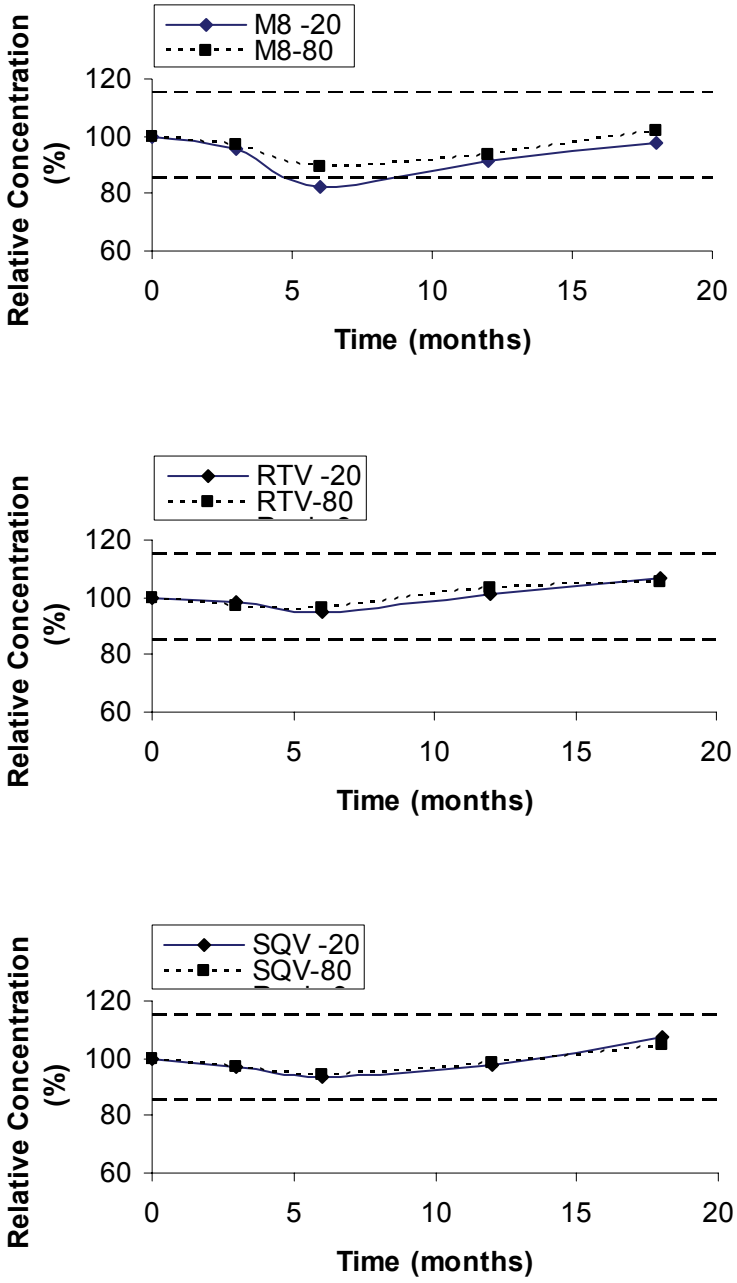


Figure 1: *Relative concentrations of the protease inhibitors in patient plasma during storage at -20°C at -80°C, mean and standard deviations, the dashed vertical lines represent the thresholds (85% and 115%)*

These calibration samples are not completely comparable to patient samples, because of the different composition. The calibration samples contain organic solvents and do not contain possible metabolites of the PIs or any comedication. In Table 2B patient samples containing nelfinavir and M8 showed degradation at -20°C[5]. This is not confirmed by our present study. The study of 2003 existed of analyzing 5 different patient samples, once at the time of blood

Table 2 A: *Acceptable stability*

Pis	M	T	Time	Rfs
NLF	SP	-20°C	4.5 months	[13]
IDV, NLF, SQV, RTV	SP	-20°C	3 months	[14]
APV, IDV, LPV, M8, NLF, RTV, SQV	SP	-20°C	6 months	[15]
APV, IDV, NLF	SP	-20°C	1 month	[16]
IDV, RTV, SQV	SP	-20°C	14 days	[17]
IDV, NLF, RTV, SQV	SP	-20°C	6 months	[18]
APV, IDV, NLF, RTV, SQV	SP	-20°C	6 months	[19]
APV, NLF, RTV, SQV	SP	-20°C	6 months	[20]
LPV	SP	-20°C	1 month	[21]
IDV, NLF, RTV, SQV	SP	-20°C, -65°C	1.5 month	[22]
APV, NLF, RTV, SQV, LPV	SP	-20°C	1 month	[23]
IDV, RTV, NLF, SQV, APV	SP	-70°C	6 months	[24]
IDV, RTV, SQV, NLF, LPV	SP	-20°C	3 months	[4]
M8	SP	-20°C	12 months	[5]
APV, LPV	SP	-20°C	4 months	[5]
IDV, NLF, RTV, SQV	SP	-20°C	2 months	[25]
IDV, APV, SQV, RTV, LPV, NLF	SP	-20°C	1 month	[26]

PIs=protease inhibitors, APV=amprenavir, IDV=indinavir, LPV=lopinavir, NLF=nelfinavir, RTV=ritonavir, SQV=saquinavir, M=material. SP=spiked plasma, T=storage temperature, Time =storage time, Rfs=references

Table 2B: *Degradation of protease inhibitors*

PIs	M	Temperature	Time	% Degradation	Refs
APV	SP	-20°C	3 months	21-24	[4]
NLF	SP	-20°C	18 months	83	[3]
NLF	PS	-20°C	18 months	30	[5]
M8	SP	-20^0C	18 months	73	[3]
M8	PS	-20°C	18 months	11	[5]
NLF	SP	-70°C	12 months	3	[3]
M8	SP	-70°C	12 months	10	[3]

PIs=protease inhibitors, M=material, SP=spiked plasma, PS=patient sample, APV=amprenavir, NLF=nelfinavir

sampling, and once after storage at -20°C for 18 months; the mean degradation was reported. In our present study the baseline concentrations and the concentrations after 3, 6, 12, and 18 months were analyzed in triplicate in contrast with the samples of 2003[5]. Obviously, the concentrations reported in this study are more reliable, because the samples were analyzed in triplicate at several time points during storage.

The results of this study show a slightly lower recovery at 6 months, which can be explained by different causes.

Any analytical assay is subject to some variation. According to the guidelines for method validation for bio-analysis of drugs[8], quality control samples are allowed to deviate 15% from the true value. Also the US Clinical Laboratory Improvement Amendments (CLIA) of 1988 allow a maximum of 20% deviation[10].

Furthermore, it is known that PIs have poor solubility in aqueous solutions[11] and their solubility is pH dependent. In general, these drugs are poorly soluble at physiological pH (7.4)[12]. Especially at low temperatures crystal formation is possible and therefore, it is of great importance to mix the samples thoroughly after defrosting. This phenomenon might be the reason for the degradation of nelfinavir and amprenavir in table 2B and also for the slightly lower recovery found at month 6 in this study.

In conclusion, the results of this study showed that the PIs amprenavir, indinavir, lopinavir, nelfinavir, M8, ritonavir, and saquinavir are stable for at least 18 months at -20°C or -80°C in plasma of HIV-infected patients.

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CHAPTER 3

False-Positive Results in Urine Drug-Screening in Healthy Volunteers Participating in Phase I Studies with Efavirenz and Rifampin

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ABSTRACT

The InstaCheck multidrug Screen Panel (Forefront Diagnostics, San Diego, CA) tested false-positive for tetrahydrocanabinol and morphine for healthy volunteers using efavirenz and rifampin respectively.

Researchers, technicians, and clinicians should be aware of the possibility of false-positive results when using the InstaCheck multidrug Screen Panel and we recommend that each laboratory evaluate rifampin and efavirenz interference with the drug screening assay in use.

Screening for drugs of abuse in urine with immunoassays is common in phase I healthy volunteer pharmacokinetic studies. This screening method is easy to perform, sensitive, and gives a quick result concerning unauthorized drug use by the healthy volunteers at entrance or during the study. However, false-positive results for 1 of the tested drugs of abuse may occur, as described in the package insert of the test panel. We observed false-positive results in urine samples from 2 different phase I studies after testing with the InstaCheck® multidrug Screen Panel. The InstaCheck® is a one-step immunoassay in which chemically labeled drugs compete for limited antibody binding sites with drugs that may be present in urine[1].

In 2 different healthy volunteer pharmacokinetic studies published elsewhere, the ENRICO study[2] and the TENORI study[3], we used the InstaCheck® multidrug Screen Panel (Forefront Diagnostics, San Diego, CA) to monitor the use of tetrahydrocannabinol, morphine, cocaine, and amphetamines. The urine drug screens were performed as outlined in the package insert[1]. No additional confirmative bioanalytical methods were executed.

In the ENRICO study, negative drug screen results were obtained for all 24 subjects on day 1 of the study and at day 10, after multiple doses of the antiretroviral protease inhibitors, nelfinavir/ritonavir 1875/200 mg once daily. On day 20 of the study, after multiple doses of nelfinavir/ritonavir + the antiretroviral nonnucleoside reverse transcriptase inhibitor efavirenz 600 mg once daily, all 24 subjects (100%) tested positive for tetrahydrocannabinol, at 14 hours after the last medication intake. Tests were repeated to ensure proper materials and operating procedures. All subjects were counseled by the study physician to exclude the use of tetrahydrocannabinol. None of the 24 volunteers reported the use of tetrahydrocannabinol.

In the TENORI study, no positive test results were seen at start and in the first part of the study after 9 consecutive doses of the antiretroviral drug tenofovir disoproxil fumarate (DF) 300 mg once daily. At the end of the second part of the study, after multiple doses of tenofovir DF and the tuberculostatic drug rifampin 600 mg once daily, 4 of 24 subjects (17%) tested positive for morphine use, at approximately 13 hours after the last medication intake. The screening was repeated a couple of hours after medication intake for 1 subject who tested positive and for 1 subject who tested negative. Both tests were positive for morphine.

The healthy volunteers who tested positive for morphine were then counseled by the study physician to exclude the use of morphine. None of the volunteers reported the use of morphine. Furthermore, all urines that tested positive with the InstaCheck® multidrug Screen Panel were repeated using Triage (Biosite Diagnostics, San Diego, CA) and tested negative.

Neither efavirenz nor rifampin cross-reactivity are mentioned in the package insert of the InstaCheck®. Both drugs and their metabolites are at least partly excreted by urine. Cross-reactivity of efavirenz with marijuana tests has been reported before[4]. The results

seen in the ENRICO study were so profound that other explanations to the observation than cross-reactivity between efavirenz and tetrahydrocannabinol are unlikely.
There are reports on the interference of rifampin with opiate immunoassays[5;6]. Our finding of 17% cross-reactivity is in line with earlier reported 12% cross-reactivity[7].
As a result of the close monitoring of the volunteers and the existing reports of similar cross-reactivity we are convinced that other explanations for the false-positive results are unlikely.

Researchers, technicians, and clinicians should be aware of the possibility of false-positive results for tetrahydrocannabinol after efavirenz use and for morphine after rifampin use, when testing with the InstaCheck® multidrug Screen Panel. Furthermore, we recommend that each laboratory evaluate rifampin and efavirenz interference with the drug screening assay in use.

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PART III

QUALITY OF TDM SERVICES

CHAPTER 4

Evaluation of Antiretroviral Drug Measurements by an Interlaboratory Quality Control Program

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ABSTRACT

Introduction: Since 1999 an international interlaboratory quality control program for analysis of antiretroviral drugs in plasma is ongoing. Results of the third round of this program are presented.

Methods: Quality control samples were prepared by spiking drug-free plasma with varying concentrations of the currently available protease inhibitors and the nonnucleoside reverse transcriptase inhibitors efavirenz and nevirapine. Thirty-three laboratories participated in the program and were requested to analyze the quality control samples.

Results: Results were from 30 laboratories. Of all measurements, 82% were performed within 80%-120% accuracy limits. Only 3 laboratories performed all their measurements within these limits, and 12 participants reported at least 90% of their analyses within the acceptance range. Mean accuracy for low drug concentrations was worse than for medium and high concentrations. The percentage of satisfactory measurements for the 6 laboratories that participated for the third time in the program increased from 54% in the first round to 85% in the third round.

Conclusions: The program revealed a large variability in the laboratories' ability to measure antiretroviral drugs accurately. This variability may have important implications for therapeutic drug monitoring of these drugs and pharmacokinetic studies. Interlaboratory testing is useful to alert laboratories to previously undetected analytical problems.

INTRODUCTION

There has been increasing interest in bio-analysis of protease inhibitors (PIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs) in recent years. Many high-performance liquid chromatographic (HPLC) assays have been published for quantitation of these drugs in plasma[1]. These analytical methods are used to study the pharmacokinetics and drug interactions of these drugs, and drug level measurements of PIs and NNRTIs are applied to individualize drug dosing (Therapeutic Drug Monitoring, TDM)[2].

In view of the wide application of bioanalytical methods for antiretroviral drugs and the clinical relevance of these applications, our department has initiated the International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring in HIV infection. The aim of this program is to alert laboratories to deviating results with respect to the analysis of PIs and NNRTIs, and thereby enable them to improve their performance.

The first round of the program was performed in 1999 and was limited to nine laboratories and to the measurement of four PIs (indinavir, nelfinavir, ritonavir, and saquinavir)[3]. The present report describes the results of the mature program, as reflected in the third round that took place in 2001. This third round of the program included 33 participating laboratories. In addition, the program was extended to the measurement of the PIs amprenavir and lopinavir and the NNRTIs efavirenz and nevirapine.

MATERIALS AND METHODS

Materials

Indinavir was obtained from Merck & Co., Inc (Rahway, NJ, USA), ritonavir and lopinavir from Abbott Laboratories (North Chicago, IL, USA), saquinavir mesylate from Hoffmann-La Roche (Basel, Switzerland), nelfinavir mesylate from Agouron Pharmaceuticals Inc. (San Diego, California, USA), amprenavir from GlaxoWellcome (Stevenage, Hertfordshire, UK), nevirapine from Boehringer (Mannheim, Germany) and efavirenz was provided by Du Pont Pharmaceuticals (Wilmington, USA). All drugs had a high purity (>97%). PIs were kept at room temperature and NNRTIs were stored at 4°C. Methanol and dimethyl sulfoxide were purchased from Merck (Darmstadt, Germany). Drug-free plasma was obtained from the regional blood bank and was stored at –20°C.

Preparation and dispatch of the quality control plasma samples

PIs were dissolved in methanol, nevirapine and efavirenz in dimethyl sulfoxide. These solvents were used because the antiretroviral drugs were soluble and stable in these fluids.

Three quality control (QC) samples were prepared by spiking plasma with three different concentrations of the PIs amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir. Three other QC samples contained the NNRTIs efavirenz and nevirapine. All concentrations (Table1) related to the active part of the chemical compound, not to the salt or esterificated form.

QC samples were dispensed in polypropylene tubes and were stored at –20°C. Stability under these and other conditions was assessed and reported previously[4-6]. QC samples were analyzed in duplicate with our own validated HPLC methods[4;6;7] as a confirmative check before samples were released for the QC program. Measurements were not allowed to deviate more than 5% from the true values.

The samples were packed on dry ice and dispatched to 33 laboratories in the United States, Europe, Canada, and Australia. Transit time of samples was 4 days at most. The laboratories were requested to analyze the QC samples within 6 weeks and return their results with details about their assays.

Data analysis

Descriptive statistics were calculated after standardization of all laboratory results to percentages with reference to the true value. By subtracting 100% from these percentages, the percentage bias from the true concentration (inaccuracy) was calculated. Twenty percent limits around the true values were considered to be appropriate threshold values for satisfactory measurements.

Analysis of variance (ANOVA) was used to evaluate the simultaneous effect of two factors, the drug to be measured and the concentration level (low, medium and high), on the absolute inaccuracy. Measurements of different drugs within the same laboratory were regarded as related to each other, and measurements of the different concentration levels of the same drug were also considered to be associated. Therefore, both drug to be measured and concentration level were repeated-measures (within subjects) factors in the analysis of variance. All statistical evaluations were performed using SPSS for Windows, (version 10.0: SPSS Inc. Chicago, Ill, USA). A p-value of < 0.05 was considered statistically significant in all analyses.

Reporting of results and sources of error

All participants were informed about their own performance and about the performance of all participants, as median inaccuracy and the range of inaccuracies were presented anonymously for all separate measurements. Results of all participants were also presented graphically.

Together with the results, an error evaluation form was sent to laboratories that reported

unsatisfactory results in one of their measurements. They were asked to complete one form for every drug with deviating results. This form categorized errors as follows (derived from similar inquiries[8,9]): methodological problems (M), technical problems (T), clerical problems (T), survey problems (S), and other (O).

RESULTS

Laboratories and analytical methods

Results were received from 30 out of 33 laboratories. Two laboratories did not report a reason for not returning results; one laboratory no longer measured antiretroviral drugs. Of the 30 responding laboratories, 28 were hospital laboratories and two were commercial laboratories. Of the 30 participants, 30% were from the United States, 60% from Europe, and the remaining 10% from Canada and Australia. All participants that provided details about their assays reported using HPLC.

Three laboratories were not able to measure the low concentrations of amprenavir, indinavir, or ritonavir, as their lower limits of quantitation were too high. Another participant reported an inability to measure the high concentration of saquinavir because this concentration was not within the range of the method.

Accuracy of measurement

A maximum number of 24 measurements (3 for each drug) were performed by the laboratories. Table 1 presents the results arranged by drug and concentration level. The performance of individual laboratories is displayed in Figure 1.

Only three laboratories reported all their results within the acceptance range (80%-120% accuracy). One laboratory (nr 30, Fig. 1) did not report any satisfactory result. Twelve out of 30 participants reported at least 90% of their results within the acceptance range. Twelve laboratories used analytical methods that appeared to have a large systematic error in one direction, as all measured concentrations of at least one drug were either above or below the 80%-120% accuracy limits.

Effect of drug to be measured and concentration level on accuracy

Descriptive analysis did not suggest large differences in mean absolute inaccuracies for measurements of the eight antiretroviral drugs (Table 1). This was confirmed by an ANOVA, which was performed for those laboratories that were able to measure all eight drugs (n=13). There was no significant main effect of the drug to be measured on the absolute inaccuracy ($F(2.783, 33.392)=0.955$, $p=0.42$). However, the concentration level to be analyzed had a

Table 1: *Results, subdivided by drug and concentration level*

Drug	N	Concentration level	(mg/L)	% Inaccuracy Median (min-max)	N and % within 80-120% range	acceptance
Amprenavir	21	Low	0.24	13 (0-100)	17/21	
		Intermediate	2.2	8 (1-65)	18/21	83%
		High	7.2	8 (2-49)	17/21	
Indinavir	27	Low	0.13	15 (1-2218)	16/27	
		Intermediate	2.3	12 (1-334)	20/27	70%
		High	11.7	11 (0-98)	21/27	
Lopinavir	23	Low	1.2	7 (1-70)	17/23	
		Intermediate	4.7	5 (1-46)	19/23	80%
		High	11.7	6 (0-44)	19/23	
Nelfinavir	28	Low	0.32	9 (2-92)	24/28	
		Intermediate	2.1	8 (0-207)	23/28	85%
		High	6.4	8 (1-61)	24/28	
Ritonavir	26	Low	0.24	7 (0-41)	16/26	
		Intermediate	2.4	9 (1-28)	23/26	81%
		High	9.7	16 (1-148)	24/26	
Saquinavir	27	Low	0.11	9 (0-446)	19/27	
		Intermediate	1.4	6 (0-35)	25/27	85%
		High	5.1	5 (0-28)	25/27	
Efavirenz	23	Low	0.46	12 (1-80)	18/23	
		Intermediate	3.7	9 (0-71)	19/23	81%
		High	6.6	8 (0-78)	19/23	
Nevirapine	18	Low	0.50	8 (2-71)	16/18	
		Intermediate	3.2	9 (1-19)	18/18	94%
		High	6.9	9 (0-24)	17/18	

Abbreviations:

N: number of measurements

Min: minimum value

Max: maximum value

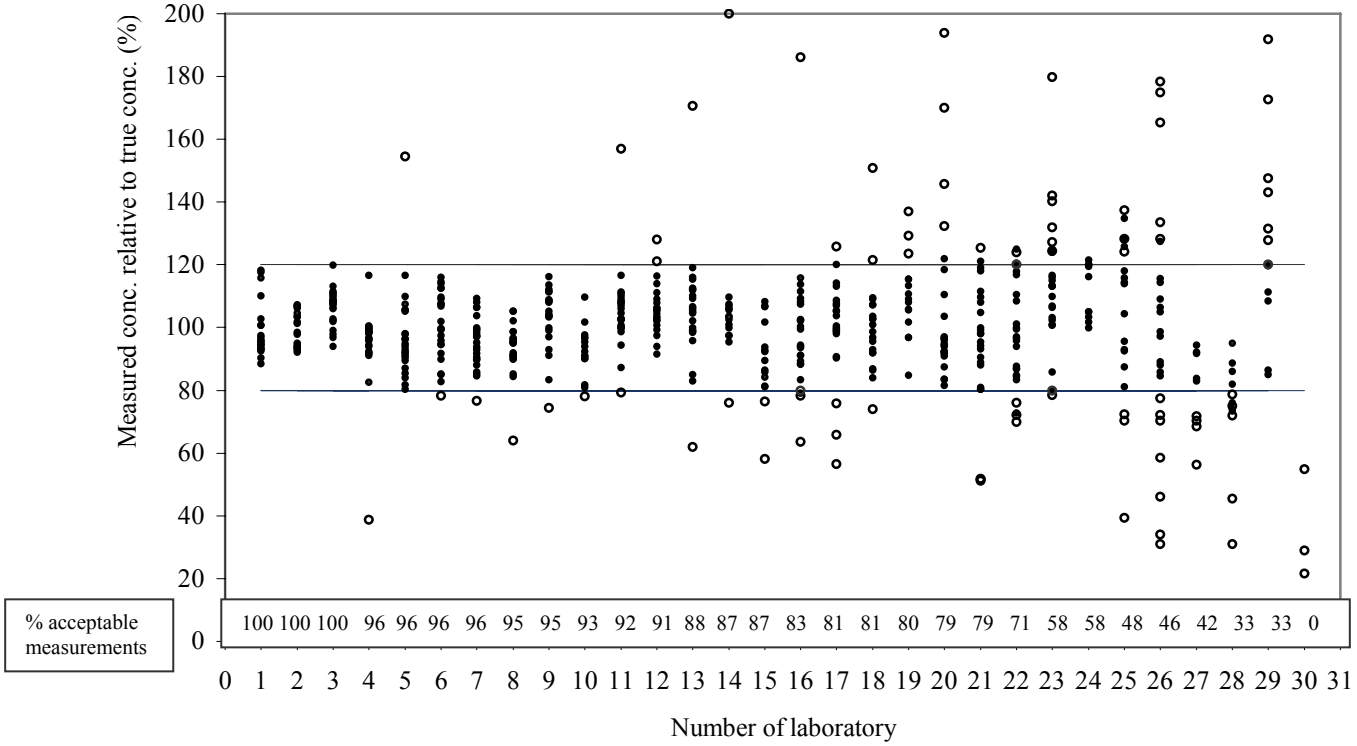

Figure 1: *Performance of individual laboratories. Diagram shows the results for all measurements arranged by laboratory. Results for individual measurements are depicted by points; the lines represent the thresholds (80%-120%)*

significant effect on the absolute inaccuracy ((F (1.035,12.421)=7.447, p=0.02). The mean absolute inaccuracy over all drugs for all 13 laboratories was 20.0% for low concentrations, 11.4% for medium concentrations, and 11.1% for high concentrations. Pairwise comparisons were performed at a Bonferroni-adjusted significance level for each separate test, keeping the overall type I-error rate at 0.05. These comparisons showed a significant difference between the absolute inaccuracies for measurements of the low drug levels versus the medium drug levels (p=0.041), and a trend towards a significant difference (p=0.06) between low level versus high level measurements.

However, no significant differences between measurements of the medium and high drug levels were observed (p=1.0). The interaction between drug to be analyzed and drug level was not significant (F (1.941, 23.294)=1.419, p=0.26).

Table 2: *Explanations for accuracies outside 80-120% fixed limits*

	Number	% of total
METHODOLOGIC PROBLEMS		
M1 Instrument problem	-	
M2 Method change before QC-program		
M3 Method not validated for all PIs	18	
M Subtotal	18	23.4
TECHNICAL PROBLEMS		
T1 Dilution error	5	
T2 Incorrect pipetting (other than dilution)	4	
T3 Misidentification of the peak	1	
T4 Calculations performed incorrectly	3	
T5 Run accepted in non-linear range		
T6 Run accepted even though controls were out of range	9	
T7 Aging stock solutions	17	
T8 Stock solutions not made of pure substances	3	
T9 Below quantitation limit	2	
T Subtotal	44	57.1
CLERICAL ERRORS		
C1 Results reported in wrong unit		
C2 Decimal point error	1	
C3 Transcriptive error into questionnaire	3	
C Subtotal	4	5.2
SURVEY		
S1 Specimen problem		
S2 Criteria for acceptance too narrow		
S Subtotal	0	0
OTHER		
O1 Unexplained/unassigned cause	11	
O Subtotal	11	14.3
Total	77	100

Sources of error

Twenty-seven participating laboratories reported at least one measurement with an inaccuracy of more than 20%. Nineteen laboratories returned their error forms.

Reported errors are presented in Table 2. Every single inaccuracy for which an explanation was reported was included in the table. There was a wide variability in explanations for deviating results. Frequent sources of error were the use of an analytical method that was not (or not properly) validated and the use of aging stock solutions.

Five out of six laboratories, that participated for the third time in this Quality Control Program, improved their performance in time. The overall percentage of acceptable measurements for these six laboratories increased from 54 % in the first round to 83% in the second round and 85% in the third round.

DISCUSSION

The results of this program show large variability in the ability of laboratories to measure antiretroviral drugs accurately. Measurement of these drugs needs to be improved in a number of laboratories that participated in the program.

The quality control program was designed to represent the reality encountered in the laboratories as close as possible. Therefore, it was decided not to use lyophilized plasma that should be reconstituted. Furthermore, no reference substances or reference plasma samples were distributed. The major difference between the QC samples and routine samples related to the presence of other drugs or metabolites, which were absent in the QC samples.

As a result of the similarities between QC samples and real samples, it can be inferred that the results of this quality program provide a measure of the rigor (or effectiveness) of the regular intralaboratory (internal) quality assurance in the participating laboratories.

On the other hand, it cannot be excluded that laboratories made extra efforts to achieve accurate results in this program[10]. This means that the results of the quality control program could also represent the best performance of the participants.

In this program, results obtained by a certain laboratory were considered acceptable if they fell within preset 80%-120% limits for accuracy. The 20% threshold was based on guidelines for method validation for bio-analysis of drugs[11], as 20% deviations are often used as a fixed criterion for inaccuracy at the lowest level of quantitation. The 20% limits are also comparable to maximal allowable error specifications for drug measurements according to the U.S. Clinical Laboratory Improvement Amendments (CLIA) of 1988[12].

The large interlaboratory variability in performance with respect to antiretroviral drug measurements may have important implications for TDM. Based on the inaccurate measurements, wrong dose adjustments might occur or patients might be advised not to adjust doses when an adjustment might be necessary. This may lead to resistance development, therapy failure, and concentration-related adverse events. In this respect, it is of special concern that low concentrations were more difficult to measure than medium or high concentrations. This is because the lowest antiretroviral drug concentrations in a dosing interval (trough concentrations) are particularly useful to measure; adequate trough levels appear to be most critical predictor for the efficacy of PIs[2].

Fortunately, the quality control program alerted the laboratories to inaccuracies and invited them to inquire possible sources of error. Our findings with respect to the possible explanations for inaccuracies (distribution over the error categories) differed from other studies[8,9,13]. In our program, the category of "technical problems" accounted for 57% of the errors (Table 2) while the studies of Hoeltge et al[9], Steindel et al[8], and Jenny et al[13] found 19%, 19% and 17% for this category respectively. This difference can be probably ascribed to the use of complex assays with difficult sample preparation that are required for analysis of antiretroviral drugs (compared to automatic assays for many other drugs). In the category of "methodological problems" all problems were caused by inappropriate validation of the assays. The participants concerned did not check their assays for interference of other PIs than the assay was developed for, while the QC samples contained all the PIs.

It seems that corrective action could prevent many errors in the future, although some failures were unexplained, (15% in our study). In fact, it appeared that the laboratories that were participating in the quality control program for the third time had better results in the third round than in the first round. It is expected that at least some of these improvements over time also affect the performance on real samples.

In conclusion, the program revealed a large variability in the performance of laboratories to measure antiretroviral drugs. The program alerted a number of laboratories to previously undetected analytical problems. This will enable them to improve their assays. In the future more agents will be included in the quality control program (e.g. nucleoside reverse transcriptase inhibitors). All laboratories measuring these antiretroviral drugs are invited to participate in this program.

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CHAPTER 5

TDM: Therapeutic Drug Measuring or Therapeutic Drug Monitoring?

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ABSTRACT

The third round of the International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring in HIV-infection (QC-program) consisted of the analysis not only of plasma samples but also of patient cases. The case was composed of different topics related to the therapeutic drug monitoring of antiretroviral drugs. The participants were asked to give recommendations concerning dose adjustments, changes to the regimen, and drug-drug interactions, to observe whether the expert recommendations were comparable. Of the 30 participants of the QC-program, 16 returned their comments and recommendations with regard to the patient case. The drug level was easy to judge, as ± 90% were able to correctly do so. Almost half of the recommendations (46%) given were satisfactory. Levels of knowledge regarding HIV treatment appeared to be variable among the respondents and for this reason were partly incomparable.

INTRODUCTION

The use of highly active antiretroviral therapy (HAART) has markedly improved the prognosis of HIV-infected patients[1]. Nevertheless, HAART can fail for various reasons, including poor adherence to therapy, toxicity, drug-drug interaction, or resistance development. Therefore, the role of therapeutic drug monitoring (TDM) in the management of antiretroviral drugs is a topic of increasing interest[2-6].

TDM has been proposed as a useful tool for the optimization of antiretroviral therapy, allowing an effective drug concentration for each individual treated. Drug concentration measurements of protease inhibitors (PIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs - applied to individualize drug dosing) are performed in a growing number of laboratories. To alert laboratories to undetected problems concerning their assays, our department has initiated an International Interlaboratory Quality Control Program for TDM in HIV infection (QC-program)[7;8]. The participating laboratories analyzed samples containing different concentrations of PIs and NNRTIs. However, not only is the accurate measurement of the drug concentration important, an expert interpretation of the concentration is absolutely critical in understanding what the results actually mean.

To observe whether the expert recommendations concerning dose adjustments, adherence, time interval between drug doses, and interaction between different drugs are comparable, the third round of the QC-program consisted of the analysis not only of plasma samples containing antiretroviral drugs but also of patient cases. The results of the analysis of the plasma samples have been reported previously[8]. The main outcome of the 30 participating laboratories that reported their results, were as follows: 82% of all measurements were performed within 80%-120% accuracy limits. Only 3 laboratories performed all their measurements within these limits; 12 participants reported at least 90% of their analyses as within the acceptance range, and the mean accuracy for low drug concentrations was worse than that for medium and high concentrations. The percentage of satisfactory measurements from the 6 laboratories participating for the third time in the QC-program increased from 54% in the first round to 85% in the third round.

The case was sent to all 30 participants of the QC-program. The results of this case are presented here.

METHODS

Case Description

The case was composed of different topics related to the TDM of antiretroviral drugs. The case consisted of a total of 3 parts and the participants were asked to give their advice at the end of each part. Patient A was an imaginary patient. Although multiple HIV drugs were prescribed for the patient, only plasma levels of PIs (nelfinavir and indinavir) and the NNRTI nevirapine were given in this case because PIs and NNRTIs satisfy many criteria for therapeutic drug monitoring, whereas nucleoside reverse transcriptase inhibitors are not suitable candidates for TDM[9]. Until now, it has not been established which pharmacokinetic parameter (e.g. minimum plasma concentration, maximum plasma concentration) should be monitored. In routine clinical care it is often difficult to draw a blood sample at a strictly defined time point; therefore, concentration ratios were used to judge the plasma levels of the PIs. The concentration ratio represents the measured plasma concentration compared with the time-adjusted average concentration, as measured in a reference population of HIV-infected individuals[10]. For nelfinavir and indinavir, a minimum concentration ratio is known below which virological failure is more likely to occur.

Patient A has started treatment with stavudine (40 mg twice daily), lamivudine (150 mg twice daily), and nelfinavir (1,250 mg twice daily). Viral load was 100,000 copies at the start of treatment, but rapidly decreased to undetectable levels at subsequent visits. The patient complains of diarrhea, which does not respond to loperamide. The HIV specialist treating patient A is concerned about subtherapeutic nelfinavir plasma levels because of the diarrhea and orders TDM for nelfinavir. A plasma sample is taken at 10 AM, 2 hours following the previous dose of nelfinavir. The nelfinavir plasma level is 3.5 mg/L.

Question 1 what is your advice?

Nelfinavir is stopped due to the diarrhea and the patient is switched to indinavir 800mg three times daily. The diarrhea disappears two days after the change, and the viral load remains undetectable. After 4 months without problems, the patient begins to complain of parasthesias in his arms and legs. He calls his general practitioner, who prescribes carbamazepine at a dose of 200 mg twice daily. Two months later, the patient visits his HIV specialist and tells him that the neuropathy has not been resolved. Additionally, his viral load has increased to 6000 copies/mL. Because of the toxicity that patient A is suffering from, the HIV specialist suspects noncompliance and orders TDM for indinavir. A plasma sample taken 4 hours after intake of indinavir contains 0.40 mg/L.

Question 2 what is your advice?

Because patient A has an insufficient virological response to the regimen, he is switched to a regimen containing zidovudine 300 mg twice daily, didanosine 400 mg once daily, and nevirapine 200 mg twice daily. After a few months, the neuropathy decreases, and carbamazepine is discontinued. Two months later, the viral load is 30,000 copies/mL. TDM for nevirapine is ordered 4 weeks later to rule out suboptimal plasma levels. The nevirapine plasma level taken 8 hours after intake is 2.9 mg/L.
Question 3 what is your advice?

The laboratories participating in rounds 1 and 2 of the QC-program were invited to participate in round 3. In addition, participants were recruited by sending emails to authors who reported methods for analyzing protease inhibitors nevirapine or efavirenz. Furthermore, participants contacted our department after the results of the first round of the QC-program were reported[7].
In rounds 1, 2 and 3, totals of 7, 18, and 30 laboratories participated, respectively. Round 3 was the first round in which a case was sent to the participants of the QC-program.

RESULTS

Results of the case were received from 16 of 30 participants who analyzed the plasma samples of the QC-program. The respondents analyzed 286 samples, 255 (86%) of which were within the acceptable limits of 80%-120% and the nonrespondents to the case analyzed 228 out of 278 (82%) within the acceptable range.
Three of the respondents were from the United States or Canada, and the other 13 from Europe. Of the total number of 16 respondents, 1 was working at a commercial laboratory, and 15 worked in university hospitals, all at the Department of Clinical Pharmacy of Pharmacology. Ten respondents were pharmacists and 6 were physicians. Of the respondents, the majority (14 out of 16) regularly published articles concerning pharmacokinetics and TDM of antiretroviral drugs and interaction studies with antiretroviral drugs in leading journals, whereas only 3 out of 14 of the nonrespondents did so.
Of the 14 participating laboratories that measured the plasma samples but did not return answers to the case, 1 participant reported not offering clinical advice. Thirteen laboratories did not give a reason for not responding to the case.

QUESTION 1
The therapeutic level of nelfinavir is found to be 3-4 mg/L 2 hours postdose[11]. Furthermore, a nelfinavir concentration ratio of less than 0.90 detects virological failures[12]. In Figure 1A, the

population curve of nelfinavir, together with the line representing the 0.90 concentration ratio, are presented. The nelfinavir level of patient A was adequate. Of the 16 respondents, 14 reported that the nelfinavir plasma level was sufficient, 1 found the level higher than expected, and 1 did not give any comment on the level.

Diarrhea is a known side effect of nelfinavir, which was noted by 4 participants. Hsyu et al[13] reported that the presence of diarrhea did not correlate with plasma nelfinavir concentrations or efficacy, although it is a common side effect of nelfinavir: 91% of the patients in the nelfinavir study of Elion[14] experienced a mild grade of diarrhea. Because the nelfinavir plasma level is not influenced in patients with diarrhea, it does not seem reasonable to adjust the dose of nelfinavir. Furthermore, 2 respondents recommended taking opium tincture or calcium supplements to treat the diarrhea, even though the diarrhea persisted during use of loperamide. Loperamide is a powerful drug against diarrhea, so changing this to opium tincture does not seem to be a logical step, although calcium supplements may help[15].

Several respondents advised to determine the C_{min} and/or the nelfinavir hydroxy metabolite M8 (M8) and reducing the dose if concentrations were sufficient. The nelfinavir level is therapeutic, so there would be no need to remeasure nelfinavir or to adjust the dose.

Finally, 8 respondents (50%) advised switching from nelfinavir to another PI if the diarrhea persisted.

Advice: Switch from nelfinavir to another PI.

QUESTION 2

An indinavir concentration ratio of less than 0.75 detects virological failures, and an indinavir level of 0.40 mg/L 4 hours postdose is low[16-18] (Fig. 1B). This was reported by 15 of the 16 respondents. With regard to the compliance of the patient, 7 respondents reported having doubts about the compliance, whereas 2 reported that they believed that the patient was compliant. One respondent reported that he did not think that the patient was compliant.

Aside from noncompliance, an interaction with carbamazepine might offer an explanation for the low indinavir level. All 16 participants noted this. Carbamazepine is a potent enzyme inducer of the CYP3A4 enzyme system, and protease inhibitors, such as indinavir, are substrates for and inhibitors of CYP3A4. So, carbamazepine may stimulate the metabolism of indinavir[19], leading to low indinavir levels and possible therapy failure[20].

Three respondents suggested giving the patient a low dose of ritonavir to neutralize the effect of carbamazepine on the low indinavir level. In such cases, the physician must pay extra attention because there can be possible interactions between ritonavir and carbamazepine, as well, leading to carbamazepine toxicity[21;22]. One respondent noted this. Ritonavir is a highly potent inhibitor of CYP3A4 and is found to interfere with carbamazepine within 12 hours of administration, leading to elevated carbamazepine levels[22].

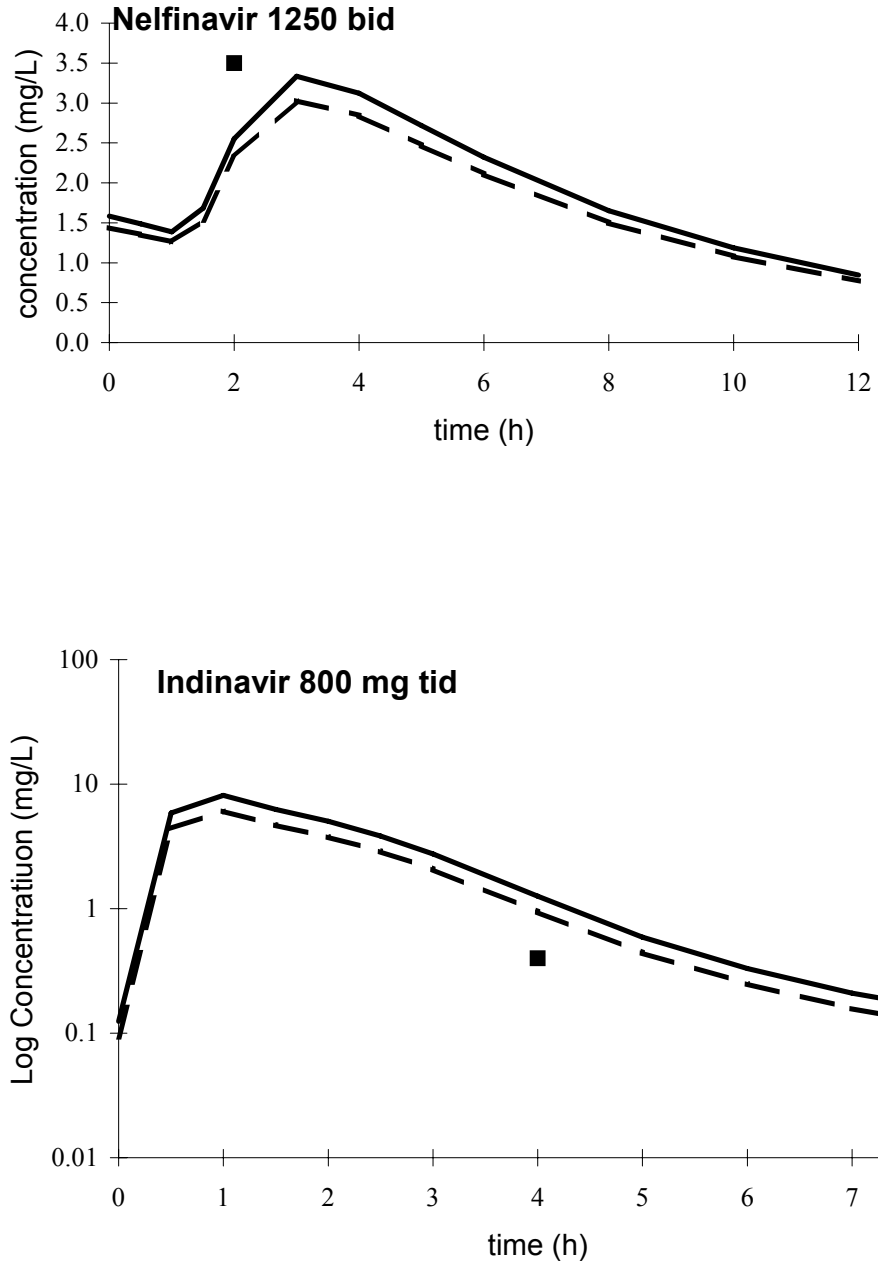

Figure 1: ***A****: Nelfinavir plasma concentrations (solid line), as a function of time after the last ingestion of nelfinavir. The curve was constructed on median values of 618 plasma concentrations obtained from 355 patients. The concentration ratio limit of 0.90 is marked by the dashed line. The concentration of patient A question 1 is reflected by* ■*.* ***B****: Indinavir plasma concentrations (solid line), as a function of time after the last ingestion of indinavir. The curve was constructed on average pharmacokinetic curves of 14 subjects. The concentration ratio limit of 0.75 is marked by the dashed line. The concentration of patient A question 2 is reflected by* ■*.*

Instead of adding a further drug interaction, a more reasonable suggestion could be to switch the carbamazepine to another drug, as suggested by 6 respondents. The drugs recommended were gabapentine, valproate, amitryptiline, clonazepam, and imipramine. Valproate seems somewhat controversial because it has been shown to stimulate the viral replication of HIV-1 in vitro[23;24].

The use of anticonvulsants, such as gabapentin and tiagabine, may be most appropriate in patients undergoing concurrent protease inhibitor therapy[25].

Three respondents suggested switching indinavir to another antiretroviral drug that does not interact with carbamazepine. This is probably not a correct recommendation, because carbamazepine did not resolve the neuropathy. Neuropathy is an adverse event of stavudine[26]. Switching stavudine to zidovudine (as suggested by 2 respondents) may not be the right advice, because of the possible cross-resistance between these 2 drugs. Lowering the dose of stavudine, as reported by 1 respondent, should not be considered. Only patients with a low body weight can be given a low dose of stavudine. The weight of the patient in this case is not specified.

The suggestion to test resistance before changing therapy was made on 2 occasions by respondents, which is a good recommendation. Youree and D'Aquila[27] reported that an increasing amount of data indicates that antiretroviral resistance testing may improve the response to therapy and increase the likelihood of achieving viral suppression.

Advice: After resistance testing, switch carbamazepine to another drug or switch therapy.

QUESTION 3

One of the 16 respondents did not answer question 3, because of a lack of experience with nevirapine.

Six respondents reported that the nevirapine level was too low or subtherapeutic, whereas 9 reported that the nevirapine level was low. The trough concentration should be above 3.4 mg/L[6;28-31] or above 3.0 mg/L, according to Vries-Sluijs et al[32]. A patient who has a level of 2.9 mg/L at 8 hours after intake will probably have a trough level even lower than this. Five respondents suggested increasing the dose of nevirapine. It is not the most obvious advice, because of the interaction of nevirapine and carbamazepine, which could occur in the first months after changing the regimen of this patient. Carbamazepine may stimulate the metabolism of nevirapine, leading to low nevirapine levels, which in turn result in resistance to nevirapine. None of the respondents noted this, although 6 respondents wondered whether resistance existed or advised performing resistance testing. This seems to be the most suitable choice.

Advice: After resistance testing, change regimen.

Table 1 presents the score per question of the respondents for the judgment of the drug level and the recommendations. It is very clear that it is more difficult to give correct advice than to judge the drug level.

Table 1: *Score per question*

	Good interpretation of drug level		Correct recommendation	
	N	%	N	%
Question 1	14/16	88	8/16	50
Question 2	15/16	94	7/16	44
Question 3	15/16	94	6/16	38
Overall	43/48	92	21/48	44

DISCUSSION

Recommendations given by the respondents were in part not comparable. This study revealed that it was more complicated to report a satisfactory recommendation than to judge the drug level correctly, these tasks having success rates of 44% and 92% respectively.

Some of the respondents did not give a recommendation or advice as requested but rather made an analysis of the case. They judged only the drug levels and mentioned the interaction between indinavir and carbamazepine without giving advice. For questions 1-3, respectively, 6, 3, and 5 respondents did not give any advice.

Furthermore, part of the advice for question 2 was to order resistance testing (2 participants). For question 3, the same 2 respondents again advised resistance testing, as did 2 other respondents. A number of studies are performed to assess the virologic and immunologic impacts of resistance testing. Considerable data from retrospective and prospective studies now support the use of HIV resistance testing[33-38]. To date, there is no unanimity as regards whether to use genotypic or phenotypic resistance assays. Both assays have their relative advantages and relative limitations[35]. Only 1 of the 4 respondents who suggested resistance testing specifically advised ordering genotype resistance testing without a stated logical reason. Nevertheless, resistance testing is increasingly becoming a basis for choosing the right regimen.

The judgment of the antiretroviral levels did not cause any trouble, as 14, 15, and 15 respondents out of a total of 16 were able to do so correctly for questions 1, 2, and 3, respectively, the success rates being 88%, 94%, and 94 % for these (Table 1). On the other hand, giving an acceptable recommendation was more complicated. For questions 1, 2, and

3, only 50%, 44%, and 38% of the respective respondents reported satisfactory recommendations. These percentages may have been different if the remaining 16 respondents had reported answers for the case. The results measuring the plasma samples of the respondents and non-respondents to the case were comparable, the acceptable results reaching totals of 86% and 82% respectively. Four of the 16 respondents reported correct recommendations for all 3 questions (data not shown).

The authors believe that TDM represents not only therapeutic drug measuring, but also therapeutic drug monitoring. Therapeutic drug measuring is only one part of TDM. Expert interpretation of a drug concentration is essential to ensure a full clinical benefit. Expert advice cannot be given without knowledge of the history of previous antiretroviral treatments, the concomitant medications, patient adherence to its treatment, the results of resistance testing in case of failure, and the correct drug level. Therefore, the authors are of the opinion that TDM should be a multidisciplinary function, where collaboration is needed among scientists, clinicians, nurses, and pharmacists.

In conclusion, the recommendations reported by the respondents are in part not comparable. On average, almost half of the recommendations given were correct. The variation in recommendations may result in implications for the patient. For example, wrong dose adjustments may occur, which may lead to resistance development. Therefore, the QC-program is of use to alert participants that TDM of antiretroviral drugs is a complex matter. The authors believe that it is of great importance that health professionals are kept well informed about the latest developments concerning TDM.

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Part IV

PHARMACOKINETIC STUDIES

CHAPTER 6

Limited Penetration of Lopinavir into Seminal Plasma of HIV-1 Infected Men

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Antiretroviral therapy can decrease the amount of HIV-1-RNA in blood plasma and in semen[1]. However, the decline of the HIV-1-RNA concentration and the evolution of virus in semen during therapy can show discordance with blood plasma, indicating viral compartmentalization[2;3]. Poor penetration into the male genital tract by some antiretroviral drugs can contribute to the different viral dynamics in this compartment[4].
Data available on drug concentrations in semen show that the penetration of the protease inhibitors (PI) nelfinavir, ritonavir, and saquinavir is poor[5]. The nucleoside analogues zidovudine, stavudine, lamivudine and abacavir, the nonnucleoside reverse transcriptase inhibitors nevirapine and efavirenz, and the PIs indinavir and amprenavir penetrate well into the male genital tract[3 6-12]. There are no data on the penetration of the PI lopinavir into the male genital tract.

HIV-1-infected men who were on a lopinavir containing regimen for a minimum of 4 weeks were recruited from our HIV outpatient clinic. The patients had to have no signs or symptoms of a genital infection. Semen samples were obtained by masturbation, centrifuged between 2 and 4 hours after collection at 1200 g for 10 minutes to obtain seminal plasma and stored at –70°C until analysis. Within 2 hours after semen collection, a blood sample was taken for the measurement of the blood plasma lopinavir and HIV-1-RNA concentrations.
The local Medical Ethics Committee approved the study, and written informed consent was obtained from all patients.

HIV-1-RNA in EDTA plasma was measured using the quantiplex bDNA assay (Bayer Corporation, Diagnostics Division, Emeryville, CA, USA), with a lower limit of quantification (LLQ) of 50 copies/mL.
HIV-1-RNA in seminal plasma was measured using the ultra Nuclisens HIV-1 QT assay (Organon Teknika, Boxtel, The Netherlands), with a LLQ of 50 copies/mL.
Lopinavir concentrations in heparinized blood plasma and in seminal plasma were measured using a high-performance liquid chromatographic (HPLC) procedure[13]. The intraday and interday variation of this assay was less than 5%.
Fourteen patients on a lopinavir containing regimen for a median of 16 weeks (range 4 – 41 weeks) were included in this study. Lopinavir was started in 9 of the patients because of virological failure on their previous antiretroviral regimen and in 5 because of side effects of their previous regimen.
The 5 patients who switched therapy because of side effects had an undetectable HIV-1-RNA in blood plasma at the moment of switching therapy. At the time the study samples were taken all 5 patients still had an undetectable HIV-1-RNA in blood plasma and an undetectable HIV-1-RNA in seminal plasma.

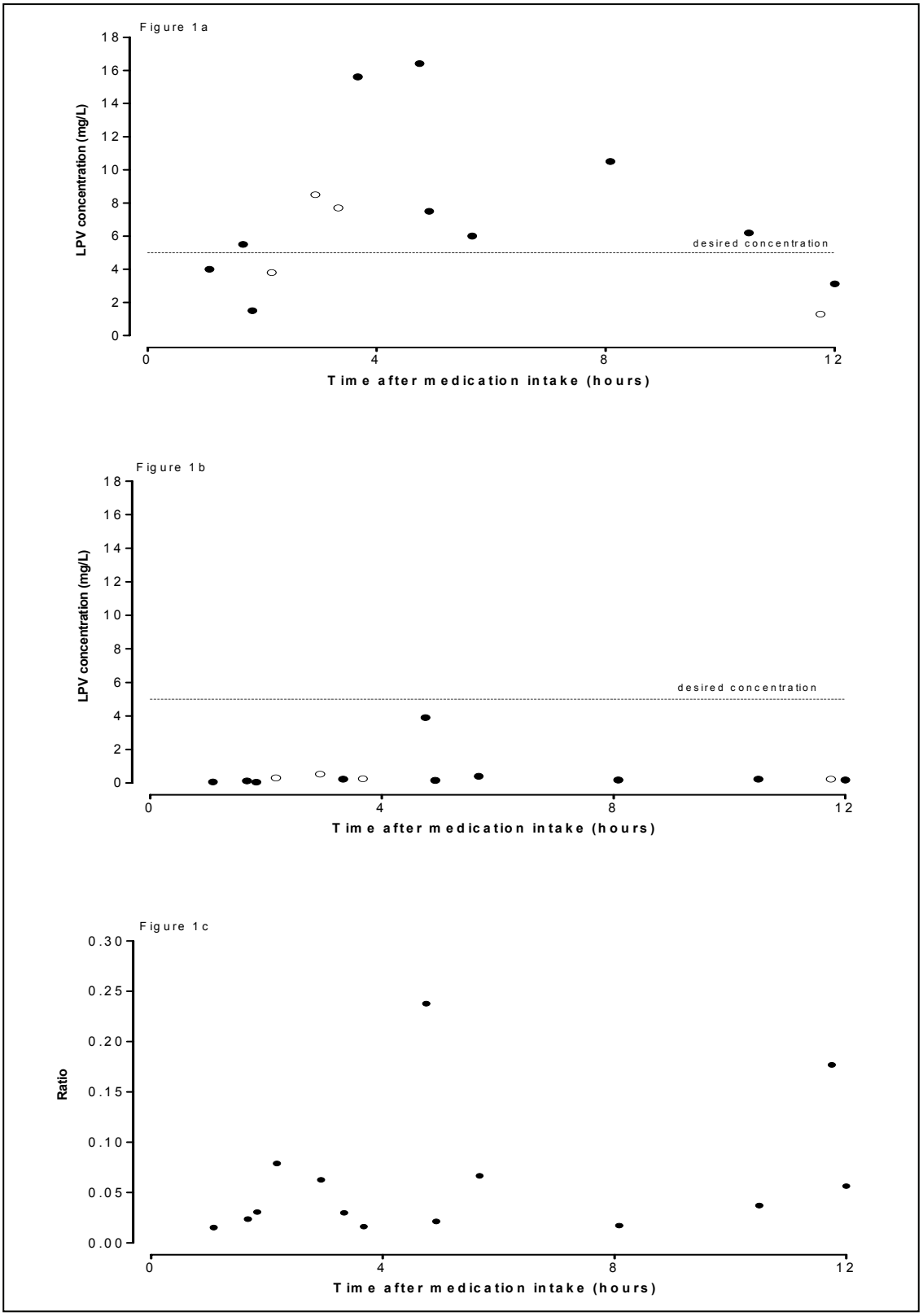

Figure 1*: Lopinavir concentrations in (a) blood plasma and (b) seminal plasma versus time after intake. O Detectable HIV-1-RNA levels in blood plasma; ● Undetectable HIV-1-RNA levels in blood plasma and seminal plasma. (c) Ratio of the concentration of lopinavir in seminal plasma and blood plasma*

Of the 9 patients starting lopinavir because of virological failure, 4 had a detectable HIV-1-RNA in blood plasma at the time the study samples were taken. These patients were not yet in a steady-state, and during follow-up their blood plasma HIV-1-RNA further decreased.
Only 1 of these 4 patients had an undetectable HIV-1-RNA in seminal plasma. The other 5 patients starting lopinavir because of virological failure had an undetectable blood plasma HIV-1-RNA. One of these patients had a detectable HIV-1-RNA in seminal plasma.

In 5 of the 14 patients the blood plasma concentration of lopinavir was below the desired concentration of 5.0 mg/L (Abbott product information). The other 9 patients had a plasma concentration > 5.0 mg/L (Fig. 1A).
The lopinavir concentration in seminal plasma ranged between 0.046 and 3.9 mg/L (median 0.23 mg/L, IQR 0.15-0.33). No relationship was found between the lopinavir concentration in seminal plasma and the time since medication intake (ρ = 0.22, p=0.45; Spearman's rank) (Fig. 1B). There was a weak relationship between the blood plasma and the seminal plasma concentration (ρ = 0.51, p=0.07;Spearman's rank). The median ratio of the concentrations of lopinavir in seminal plasma and in blood plasma was only 0.034 (IQR 0.021-0.070) (Fig. 1C). There was no relationship between the lopinavir concentration in blood plasma or seminal plasma and HIV-1-RNA blood plasma or in seminal plasma (ρ =0.11, p=0.73; Spearman's rank) (Fig. 1A and 1B) and no relationship between the lopinavir concentration in seminal plasma and HIV-1-RNA in seminal plasma (ρ=0.46, p= 0.11; Spearman's rank).
We demonstrated that lopinavir has a poor penetration into the seminal plasma, with a median concentration of only 0.23 mg/L (range 0.046-3.9 mg/L), assuming the same percentage protein binding in seminal plasma as in blood plasma (98-99%; Abbott product information). Because of this poor penetration one would expect a poor suppression of the viral replication in semen. However, only 4 out of the 14 patients had a detectable HIV-1-RNA in seminal plasma of which 3 patients were not yet in a steady-state. An explanation for this could be that all patients were on a regimen containing at least one other antiretroviral drug with a good penetration into the seminal plasma (data not shown). It is, however, conceivable that the replication of HIV-1 in the genital tract of our patients is only partially suppressed by the other drugs of the regimen, usually 2 nucleoside analogues. Although most of the patients had an undetectable HIV-1-RNA level in seminal plasma, the median time on lopinavir was only 16 weeks, and it is possible that in time there will be a selection of HIV-1 strains resistant for the other drugs used. There are indications that the selection of resistant HIV-1 strains in the male genital tract can differ from that in blood plasma[14]. If resistant HIV strains migrate to other compartments a patient is at risk for systemic virological failure. Resistant strains in seminal plasma may also lead to the infection of other

persons with resistant strains. A longer follow-up of HIV-1-RNA in seminal plasma is necessary to be confident that selection of resistance mutations is not a risk of regimens with only partial penetration into the male genital tract.

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CHAPTER 7

Pharmacokinetic Study of Tenofovir Disoproxil Fumarate Combined with Rifampin in Healthy Volunteers

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ABSTRACT

Tenofovir disoproxil fumarate (tenofovir DF) was studied in combination with rifampin in 24 healthy subjects in a multiple-dose, open-label, single-group, two-period study. All subjects were given tenofovir DF at 300 mg once a day from days 1 to 10 (period 1). From days 11 to 20 the subjects received tenofovir DF at 300 mg combined with rifampin at 600 mg once daily (period 2). The multiple-dose pharmacokinetics of tenofovir (day 10 and 20) and rifampin (day 20) were assessed. The drug-related adverse events (AEs) experienced during this study were mostly mild. Only one grade 3 AE possibly or probably related to the treatment (raised liver enzyme levels) occurred during period 2; the subject was withdrawn from the study. Pharmacokinetic data for 23 subjects were thus evaluable. Point estimates for the mean ratios of tenofovir with rifampin versus tenofovir alone for the area under the concentration-time curve from zero to 24h (AUC_{0-24}), the maximum concentration of drug in plasma (C_{max}), and the minimum concentration of drug in plasma (C_{min}) were 0.88, 0.84, and 0.85 respectively. The 90% classical confidence intervals for AUC_{0-24}, C_{max}, and C_{min} were 0.84 to 0.92, 0.78 to 0.90, and 0.80 to 0.91, respectively, thus suggesting pharmacokinetic equivalence. Similarly, coadministration of rifampin and tenofovir DF did not result in changes in the values of the tenofovir pharmacokinetic parameters. For rifampin, the values of the pharmacokinetic parameters found in this study were comparable to those found in the literature, indicating that tenofovir DF has no effect on the pharmacokinetics of rifampin. In conclusion, adaptation of either the rifampin or tenofovir DF dose for the simultaneous treatment of tuberculosis and human immunodeficiency virus (HIV) infection in HIV-infected patients is probably not required.

INTRODUCTION

Co-infection with *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) is frequent, particularly in Africa and Asia[1-3]. Simultaneous treatment of tuberculosis and HIV-infection may lead to complex combination therapy. Rifampin is a drug of choice for the treatment of tuberculosis. Rifampin is known to have major pharmacokinetic interactions with HIV protease inhibitors and nonnucleoside reverse transcriptase inhibitors[4-9]. Tenofovir disoproxil fumarate (tenofovir DF) is the first drug from a new class of anti-HIV agents (nucleotide reverse transcriptase inhibitors) that has been recently approved for use for the treatment of HIV-infection in adults. However, no data are available regarding its pharmacokinetics in combination with tuberculostatic drugs, in particular, rifampin. No influence of rifampin on the pharmacokinetics of tenofovir is expected, because both drugs are metabolized and eliminated in different ways. Tenofovir is eliminated unchanged by glomerular filtration and active tubular secretion[10;11], while rifampin is extensively metabolized by intestinal and hepatic metabolism[12]. However, pharmacokinetic interaction cannot be excluded.

This clinical trial described here was designed to explore the pharmacokinetics of tenofovir DF with and without rifampin in an effort to establish whether there is a need to adjust the dosage of either medication when the two medications are used for the treatment of patients coinfected with *M.tuberculosis* and HIV.

MATERIALS AND METHODS

Study Design

The present study was designed to evaluate the effect of 600 mg of rifampin on the pharmacokinetics of 300 mg of tenofovir DF and also to assess whether tenofovir DF has a substantial impact on steady-state exposure to rifampin. This study was a multiple-dose, open-label, single-group, two-period study with 24 healthy volunteers. First, the subjects received tenofovir DF at 300 mg once daily for 10 days (period 1). At study day 10, a steady-state 24-h pharmacokinetic curve was obtained for tenofovir. During the second period of the study (period 2), tenofovir DF at 300 mg was combined with rifampin at 600 mg once daily, again for 10 days. At study day 20, 24-h steady-state pharmacokinetic curves were obtained for tenofovir and rifampin. During the study both tenofovir DF and rifampin had to be taken with breakfast. On the days prior to study days 9 and 19, the subjects reported to the study center for direct observation of dosing with the medications with a standardized breakfast. Subsequently, on the evenings of study days 9 and 19 the subjects remained at the study

center for 2 overnight stays and remained at the study center until the mornings of study days 11 and 21, respectively. On days 9, 10, 11, 19, and 20 the subjects received a standardized breakfast of 550 kcal (two slices of white bread, 15 g low fat margarine, 14 g jelly, 150 mL orange juice and 150 mL skim milk). The medication was administered immediately after breakfast with 200 mL of tap water. All other meals and snacks on the pharmacokinetic study days were also standardized. When the subjects took the medication at home, study drugs were administered with breakfast (at least two and at most three slices of wheat bread).

No crossover design was used in this study, because rifampin could lead to considerable carryover effects, due to its long-lasting cytochrome P450-inducing effect. To eliminate this effect a longer washout period would be necessary, but this would have significantly prolonged the duration of the study and would have led to difficulties with subject recruitment and retention. This study was reviewed and approved by the independent ethics committee Arnhem-Nijmegen. Written informed consent was obtained from each study subject prior to the conduct of any study related activity.

Study subjects

Twenty-four healthy male and female subjects were eligible for inclusion in the study. The subjects could be between 18 and 65 years of age with a body weight of at least 50 kg and in a good age-appropriate health condition, as established by the individual's medical history; a physical examination; electrocardiography; and the results of biochemistry, hematology and urinalysis within the 3 weeks prior to administration of the first dose. Other inclusion criteria were an ability to sign informed consent voluntarily and a willingness to refrain from the use of contact lenses during treatment with rifampin. Exclusion criteria were as follows: positive tests for HIV, hepatitis B virus, or hepatitis C virus; a tuberculin skin test reaction of more than 15 mm or a tuberculin skin test reaction of 1 to 15 mm with a chest X ray with abnormalities consistent with tuberculosis; pregnancy; breast-feeding; the lack of adequate contraception (e.g., hysterectomy; bilateral tubal ligation; the use of an intra-uterine device, total abstinence, or double-barrier methods; or a postmenopausal state for 2 years) among female subjects of childbearing potential; a creatinine clearance rate < 60 mL/min; or a serum creatinine level above 133 µmol/L.

Sampling for pharmacokinetic studies

For determination of the tenofovir and rifampin concentrations in blood plasma, samples of 5 mL blood, recovered to obtain at least 2 mL of plasma, were collected in heparinized hard plastic tubes at the following times: just before drug intake (predosing); on day 10 and day 20; and at 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, and 24 h after drug intake. The blood samples were

centrifuged at 2,500 x g for 10 minutes at 4°C. The plasma was divided into equal portions, transferred to polypropylene tubes, and stored at ≤ -18°C for samples containing tenofovir and ≤ -70°C for samples containing rifampin.

Safety

Blood samples for serum biochemistry analyses, including tests for glucose and hematological analyses, and urine samples for urinalysis were taken on study days 1, 4, 9, 11, 15, 19, and 21. These samples were taken while the subjects were in a fasting condition. In females of childbearing potential, testing of blood for human chorionic gonadotropin was performed at the screening visit and on study days 11 and 21. An instant test of urine for human chorionic gonadotropin was performed on study day –1. To avoid possible interactions between drugs of abuse and study drugs, a urine drug screen was performed at the screening visit and on study days –1, 9, and 19 with the Instacheck™ Multi-Drug Screen panel (Forefront Diagnostics, San Diego, Calif.). Vital signs for cardiovascular safety (systolic and diastolic blood pressure and heart rate) were monitored, and an electrocardiogram was recorded at the screening visit. The medical and nursing staff of the trial center monitored the subjects for adverse events (AEs) throughout their confinement. Subjects voluntarily reported any AE or reported AEs in response to general questioning. All AEs occurring between the first intake of the trial medication(s) and the end of the trial were reported. The relationship of the trial drug(s) was not related or unlikely to be related to the trial drug(s) if evidence existed that the AE had a source other than the trial drug(s). AEs were recorded as possibly or probably related to the trial drug(s) if a temporal relationship existed between the event onset and administration of the trial drug(s) and there was no evidence of an alternative cause for the event.

The severity of the AEs were recorded and graded according to the common toxicity criteria (grade: 1, 2, 3, and 4) of the National Institute of Allergy and Infectious Diseases.

Bio-analysis

Tenofovir concentrations were determined by using a validated high-performance liquid chromatography assay with a fluorimetric detector by a modified method[13]. A Symmetry Shield RP18 analytical column (3,5 µm; 150 by 4.6 mm; Waters, Etten-Leur, the Netherlands) was used. The method involved extraction of the drug and internal standard, adefovir (Gilead Sciences, Foster City, Calif), from 100 µL human plasma by adding 200 µL of acetonitrile. The supernatant was evaporated, and 200 µL of 0.34% chloroacetaldehyde in 50 mM acetate buffer (pH 4.5) was added. Fluorescent compounds were obtained by 40 minutes of incubation at 90°C. After the samples were cooled at –20°C for 5 minutes, 10 µL was injected onto the column. The flow rate was 1 mL/min. The mobile phase consisted of a

mixture of phosphate buffer (50 mM; pH 6.8)/acetonitrile (96:4; vol/vol) that resolved the drug and the internal standard from endogenous matrix components and other drugs that were possibly present. Chromatographic analysis was performed at 30°C under isocratic conditions with extinction and emission wavelengths of 232 and 420 nm, respectively. The retention times of tenofovir and the internal standard, adefovir, were 6.34 and 3.90 minutes, respectively. The concentrations of the quality controls used were 0.03, 0.21, and 1.05 mg/L. The intra- and interassay coefficients of variation were less than 4% for all quality controls. The lower limit of quantification was 0.0045 mg/L. The rate of recovery of tenofovir from human plasma was 86%.

Rifampin concentrations were determined by using a previously described[6] high-performance liquid chromatography method. The concentrations of the quality controls used were 2.85, 9.5, and 24 mg/L. The intra- and interassay coefficients of variation were less than 1.1% for all quality controls. The lower limit of quantification was 0.50 mg/L. Samples of the same subjects were analyzed by use of the same standard curve.

Pharmacokinetic analysis

Pharmacokinetic parameters for tenofovir and rifampin were calculated by noncompartmental methods by use of the WinNonlin software package (version 4.1; Pharsight Corporation, Mountain View, Calif.) and the log/linear trapezoidal rule. On the basis of the individual plasma concentration-time data, the following pharmacokinetic parameters were determined: the area under the plasma concentration-time curve (AUC) from time zero to 24 h (AUC_{0-24}; in milligram.hour per liter), the maximum concentration of drug in plasma (C_{max}; in milligrams per liter), the time to reach C_{max} (T_{max}; in hours), the minimum concentration drug in plasma (C_{min}; in milligrams per liter), the apparent elimination half-life ($t_{1/2}$; in hours), and the apparent oral clearance (CL/*F*; in liters per hour). AUC_{0-C^*}, where C* is the last quantifiable concentration, was calculated for rifampin. C_{min} and CL/*F* were not calculated for rifampin.

Statistical analysis

Statistical analyses were performed with SPSS software (version 11.0; SPSS Inc. 1989 to 1999). Descriptive statistics were calculated with Excel 2000 software (Microsoft Corporation 1985 to 1999). Evaluation of the AUC_{0-24} and the C_{max} of tenofovir was the main objective of this trial. These parameters are considered the primary characteristics for the extent and the rate of drug absorption, respectively. The bioequivalence of tenofovir was determined by comparing the values of the relevant pharmacokinetic parameters obtained with the test treatment (tenofovir DF and rifampin on study day 20) to those obtained with the reference treatment (tenofovir DF alone on study day 10) by using the following statistical methods. The AUC_{0-24}, C_{max}, and C_{min} of tenofovir were reported for study day 10 and study day 20

together by use of the ratios of the values on study day 20/values on study day 10. The arithmetic means and standard deviations are given for study day 20 and study day 10. The geometric mean ratios and 90% classical confidence intervals for AUC_{0-24}, C_{max}, and C_{min} were calculated. Treatments were considered bioequivalent if the respective 90% classical confidence intervals for AUC_{0-24} and C_{max} were included within the bioequivalence range of 80% to 125%[14]. The values of the pharmacokinetic parameters for rifampin were compared with data from literature by the use of descriptive statistics. The study was powered for the tenofovir C_{max} by using nQuery software, and a sample size of 15 was required to achieve an 80% power to reject the null hypothesis that the two treatments are not equivalent in favor of the alternative hypothesis that the means of the two treatments are equivalent, when the expected difference is 0.000. By this approach, a sample size of 15 would provide a 93% power for AUC_{0-24}. By considering the possibility that the subjects would drop out and/or that some difficulties with sample or pharmacokinetic analysis with some subjects would occur, 24 subjects were enrolled in this study.

RESULTS

Demographics

Twenty-four subjects (13 males, 11 females) were enrolled in this trial. One male subject was black; all other subjects were Caucasian. The mean age of the subjects was 41 years (range, 20 to 63 years). The mean body weight was 77 kg (range, 58 to 97 kg), and the mean height was 1.75 m (range, 1.59 to 1.88 m).

Pharmacokinetics

The pharmacokinetic evaluation was based on data sets for subjects that completed the study on both study days (study days 10 and 20). Data for 23 subjects were included in the pharmacokinetic analysis of tenofovir and rifampin. Table 1 provides a summary of the values of the pharmacokinetic parameters for tenofovir, including the arithmetic means, geometric mean ratios, and 90% confidence interval estimates for the pharmacokinetic parameters for tenofovir alone (study day 10) and tenofovir in combination with rifampin (study day 20). The tenofovir AUC_{0-24}, C_{max}, and C_{min} were lower in period 2 when tenofovir DF was coadministered with rifampin. However, the magnitudes of these differences were small, with geometric mean ratios (90% confidence intervals) of 0.88 (0.84 to 0.92), 0.84 (0.78 to 0.90), and 0.85 (0.80 to 0.91) for AUC_{0-24}, C_{max}, and C_{min}, respectively, suggesting pharmacokinetic equivalence when tenofovir DF was dosed with or without rifampin.

Table 1: *Pharmacokinetics of tenofovir*

Parameter	Day 10	Day 20	Geometric mean ratio day20/day10 and 90% CI
AUC_{0-24} (mg.h/L)	3.56 ± 0.77 (3.48)[a]	3.11 ± 0.57 (3.06)	0.88 (0.84-0.92)
C_{max} (mg/L)	0.36 ± 0.080 (0.36)	0.30 ± 0.060 (0.30)	0.84 (0.78-0.90)
C_{min} (mg/L)	0.071 ± 0.016 (0.069)	0.060 ± 0.011 (0.059)	0.85 (0.80-0.91)
T_{max} (h)[b]	1.0 (1.0-3.0)	1.0 (1.0-2.0)	
$T_{1/2}$ (h)	13.8 ± 4.53 (13.2)	11.6 ± 2.77 (11.2)	
CL/F (L/h)	88.1 ± 19.0 (86.2)	99.8 ± 20.3 (98.0)	

N=23, CI= confidence interval

[a] Values are arithmetic means ± standard deviations (geometric means), unless indicated otherwise. [b] Values are medians (ranges)

Table 2 *Pharmacokinetics of rifampin*

	Value on day 20 (this study) (n=23)	Literature (with breakfast) (n=14)[a]
T_{max} (h)	2.4 (0.6)[b]	4.43 (1.11)
C_{max} (mg/L)	10.9 (3.0)	7.27 (2.25)
AUC_{0-12} (mg.h/L)	43.27 (15.28)	50.97 (14.27)
$T_{1/2}$ (h)	1.5 (0.3)	

[a] The data are from reference 15 and are for subjects who received rifampin with breakfast.

[b] Values are means (standard deviations)

Figure 1 illustrates the effects of rifampin on the mean concentration-time profiles of tenofovir. Table 2 presents the values of the pharmacokinetic parameters for rifampin when it was combined with tenofovir and the values of the pharmacokinetic parameters of rifampin from literature[12;15]. The values of the pharmacokinetic parameters of rifampin when it was combined with tenofovir are comparable to those in the literature when rifampin is administered with food, suggesting that tenofovir has no influence on rifampin exposure.

Safety

All 24 subjects reported one or more AEs at some time during the study. No subject experienced a grade 4 AE or a serious AE. In total, 160 grade 1 or grade 2 AEs were

reported. A total of 102 AEs were judged to be possibly or probably related to a study drug(s). During treatment with tenofovir DF (period 1), 33 possibly or probably related AEs were reported, while during period 2 (tenofovir DF combined with rifampin) 69 possibly or probably related AEs were reported. Of the 69 AEs reported during period 2, 24 AEs were related only to rifampin. Each subject reported discoloration of the urine. Most of the study-drug related AEs were mild (85% were grade 1 in severity). All AEs resolved after withdrawal of treatment.

All 24 subjects completed treatment period 1 (tenofovir DF alone). The most common AEs that were reported during treatment with tenofovir DF were fatigue, headache and gastrointestinal disorders.

During period 2 one subject was withdrawn from the study due to several complaints, which were rash, headaches, abdominal disorders, fatigue, somnolence, and dizziness. The study medications were stopped on study day 15. At the follow up visit, 5 days later, the subject developed elevated liver enzymes levels, which were judged to be a grade 3 AE. Nine days after the first follow-up visit the liver enzyme levels returned to normal. The AEs that occurred during the combination treatment with tenofovir DF and rifampin consisted mainly of flu-like symptoms (e.g. fatigue, headache and gastrointestinal disorders) and urine discoloration, which are well-known AEs of rifampin[16].

No clinically significant hematology or urinalysis values were observed in this study.

DISCUSSION

This study was designed to investigate whether rifampin influences the pharmacokinetics of tenofovir. The study showed that bioequivalence could be suggested for tenofovir DF combined with rifampin and tenofovir DF given alone and that the combination of tenofovir DF with rifampin was generally well tolerated, as only one patient prematurely discontinued from study.

The confidence intervals for AUC and C_{min} were 0.84 to 0.92 and 0.80 to 0.91, respectively, while the confidence interval was 0.78 to 0.90 for C_{max}. By definition, bioequivalence was proven for AUC and C_{min}, but was only suggested for C_{max}[14].

The tenofovir DF dose used in this study (300 mg once daily) is the dose recommended for the treatment of HIV infection in adults[17]. The rifampin dose used (600 mg once daily) is an accepted regimen for the treatment of tuberculosis in patients weighing more than 50 kg[16].

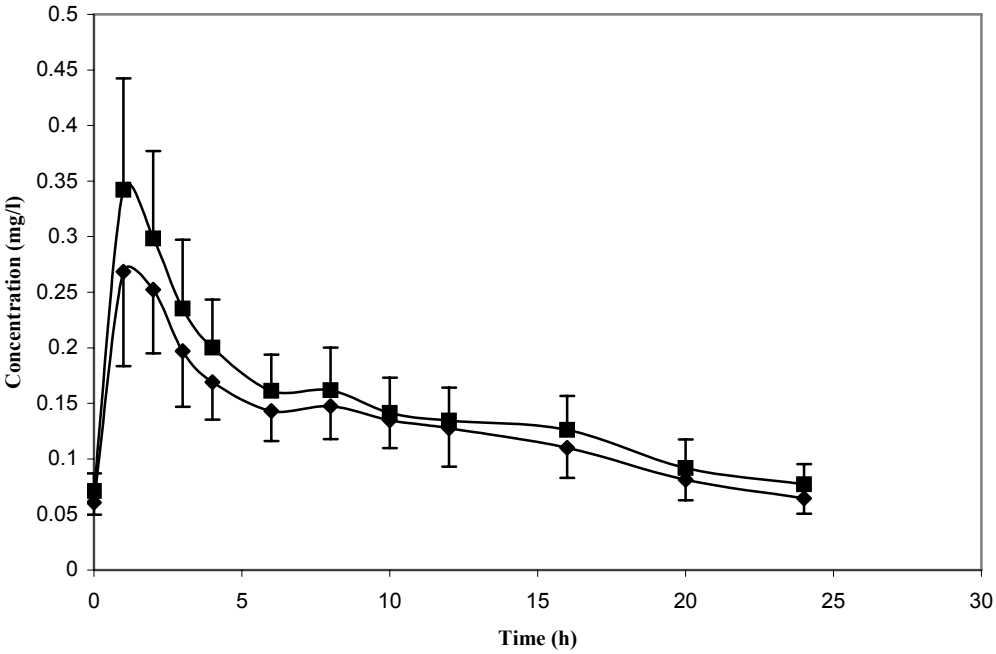

Figure 1: *Plasma tenofovir concentrations ■ tenofovir concentration on study day 10 (n=23) after administration of 300 mg once daily, ♦ tenofovir concentrations on study day 20 (n=23) after administration of 300 mg combined with rifampin at 600 m*g *once daily. Data are presented as means, and error bars indicate standard deviations*

A previous study[18] has shown that steady-state conditions for rifampin are generally achieved after the sixth daily dose of rifampin at 600 mg. To ensure the achievement of steady-state pharmacokinetics, subjects were given tenofovir DF combined with rifampin for 10 days before pharmacokinetic assessment.

The reason for the lower observed tenofovir levels is unknown. Several mechanisms could contribute to this interaction. Because tenofovir is not metabolized and is eliminated unchanged by a combination of glomerular filtration and active tubular secretion[10;11], it is unlikely that the inducing effect of rifampin on hepatic and intestinal cytochrome P450 enzymes (especially CYP3A4)[4] is the mechanism responsible for this effect. This is supported by no apparent changes in tenofovir $t_{1/2}$ and no clinically relevant effects of rifampin on the tenofovir C_{min}.

Similarly, as tenofovir minimally binds to proteins in human plasma or serum (< 0.7% and 7.2% respectively)[17], altered distribution is also probably not the mechanism responsible for the pharmacokinetic differences observed. As the decrease in tenofovir C_{max} was 16% while the decrease in AUC_{0-24} was 12%, the cause may be in the process of tenofovir DF or

tenofovir absorption. Rifampin has been shown to be an inducer of the efflux transporter P glycoprotein[19]. No information exists in the literature that P glycoprotein plays a role in the process of absorption of tenofovir in vivo. However, van Gelder et al[20] have described the transport of tenofovir DF by a P-glycoprotein-related efflux mechanism in the Caco-2 system.
AEs led to one discontinuation in this study; grade 3 elevations in hepatic enzyme levels were reported after the medication was stopped during period 2, when tenofovir DF was combined with rifampin. Liver disturbance is a well-known side effect of rifampin. Gastrointestinal disorders are well-known AEs of both tenofovir and rifampin and occurred in a total of 46% of the study subjects during both study periods. During period 2 all subjects reported discoloration of their urine which is a well-known AE of rifampin[16].
Some additional considerations are important for the extrapolation of the results of this study to patients. First, it should be noted that all the participants in this study were healthy subjects. It cannot be excluded that the pharmacokinetics of tenofovir and rifampin are different in HIV-infected patients coinfected with *M. tuberculosis* due to one or both of the diseases. Second, 23 of the 24 subjects of this study were Caucasian. Race might have an effect on the values of the pharmacokinetic parameters of tenofovir, although the available pharmacokinetic data do not indicate substantial differences with regard to race[17]. Finally, the subjects in this study were given tenofovir DF and rifampin only, while HIV-infected patients coinfected with *M. tuberculosis* are treated with other antiretroviral and tuberculostatic drugs, which can cause interactions.

In conclusion, the data from this study demonstrate that the addition of rifampin to tenofovir DF is well tolerated, and the small decrease in plasma tenofovir levels during combination treatment suggests that these drugs can be coadministered without the need for dose adjustments. This implies that standard doses should be a starting point for using these medications in HIV-infected patients. Additional pharmacokinetic studies in a clinical setting are warranted to confirm the findings of this study.

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CHAPTER 8

Assessment of Drug-Drug Interactions Between Tenofovir Disoproxil Fumarate and the Nonnucleoside Reverse Transcriptase Inhibitors Nevirapine and Efavirenz in HIV-Infected Patients

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ABSTRACT

Introduction: Tenofovir disoproxil fumarate (DF) has been studied in combination with efavirenz in healthy volunteers and no interaction was found. No data are available on the possible interaction of tenofovir DF with nevirapine and efavirenz in HIV-infected patients. In this study the combination of nevirapine 200 mg twice daily with tenofovir DF 300 mg once daily and nevirapine 400 mg once daily with tenofovir DF 300 mg once daily were compared with nevirapine twice daily or once daily without tenofovir DF in HIV-infected patients. Furthermore, the combination of efavirenz 600 mg and tenofovir DF 300 mg once daily was compared with use of efavirenz 600 mg once daily only.

Methods: Data were retrospectively collected from routine therapeutic drug monitoring plasma samples. Nevirapine, efavirenz, tenofovir plasma levels, and tenofovir concentration ratios were analyzed. The concentration ratio represents the measured plasma concentration compared with the time-adjusted average concentration, as measured in a reference population. Six different groups were studied: 200 mg nevirapine twice daily, 400 mg nevirapine once daily, 600 mg efavirenz once daily, all without tenofovir DF (group 1, 2, and 3, respectively), and the same groups combined with tenofovir 300 mg once daily (group 4, 5, and 6, respectively).

Results: Plasma samples were evaluable for 272, 18, 126, 32, 94, and 118 patients in the groups 1-6 respectively. No differences were found in plasma levels for tenofovir, nevirapine, and efavirenz for either of the combinations studied. Addition of tenofovir DF to efavirenz or nevirapine in HIV-infected patients does not influence the plasma levels of nevirapine or efavirenz. Furthermore, nevirapine and efavirenz have no effect on tenofovir plasma levels and tenofovir concentration ratios.

Conclusion: Efavirenz or nevirapine can be coadministered with tenofovir DF in HIV-infected patients without dose modifications.

INTRODUCTION

Current treatment of patients with human immunodeficiency virus (HIV) infection involves the use of highly active antiretroviral therapy regimens, which generally comprise at least 3 drugs belonging to at least 2 of the currently available antiretroviral classes[1]. Tenofovir disoproxil fumarate (DF) is the first drug from a new class of HIV agents (nucleotide reverse transcriptase inhibitors) that is approved for use in the treatment of HIV infection in adults[2]. Because tenofovir is not metabolized but eliminated unchanged by a combination of glomerular filtration and active tubular secretion[3;4] and tenofovir does not inhibit drug metabolism mediated by any of the following CYP450 isoforms: CYP3A4, CYP2D6, CYP2C9, or CYP2E1[2], it was thought not likely to cause interactions with other HIV agents. Although tenofovir is not involved in clinically significant drug-drug interactions with the majority of other antiretrovirals and frequently used other medications in HIV-infected patients, there are a few notable exceptions. Unexpectedly, tenofovir has been found to increase plasma exposures of didanosine, putatively via inhibition of its metabolism by purine nucleoside phosphorylase; reducing the didanosine dose from 400 mg once daily to 250 mg once daily in patients weighing more than 60 kg is recommended during concomitant use. In addition, tenofovir has been shown to decrease atazanavir plasma levels in pharmacokinetic studies in healthy subjects via an unknown mechanism; use of ritonavir-boosting (atazanavir 300 mg with 100 mg of ritonavir) is recommended during concomitant use.

Of the nonnucleoside reverse transcriptase inhibitors, efavirenz was studied in combination with tenofovir DF in healthy volunteers[5]. Although there was no effect of tenofovir DF on the pharmacokinetics of efavirenz and no alterations in the pharmacokinetics of tenofovir were observed, it is prudent to confirm the absence of a drug-drug interaction between tenofovir DF and efavirenz in HIV-infected patients. In addition, no data are currently available regarding the pharmacokinetics of another nonnucleoside reverse transcriptase inhibitor, nevirapine, in combination with tenofovir DF. Possible interactions between tenofovir and nevirapine or efavirenz could involve decreased or increased level of one of the drugs, resulting in subtherapeutic concentrations or toxic concentrations of the antiretroviral drugs, respectively. One useful mechanism to assess potential drug-drug interactions is through exploration of large therapeutic drug monitoring (TDM) databases containing data from HIV-infected patients.

The primary objective of this study was to investigate the effect of tenofovir DF on the plasma nevirapine levels in HIV-infected patients. The secondary objective was to explore the effect of nevirapine on tenofovir plasma levels. In addition, the effect of tenofovir DF on efavirenz pharmacokinetics (and vice versa) was assessed to validate this TDM database analysis approach and to confirm results obtained from a healthy volunteer pharmacokinetic study[5].

METHODS

Study design

This was a retrospective study that consisted of 6 groups of HIV-infected patients. Three control groups (1, 2, and 3) and 3 tenofovir DF groups (4, 5, and 6) were composed for comparing nevirapine and efavirenz data. The patients in the control groups were using nevirapine 200 mg twice daily (group 1), nevirapine 400 mg once daily (group 2) or efavirenz 600 mg once daily (group 3), each without tenofovir DF. These were compared with groups using tenofovir DF 300 mg once daily combined with nevirapine 200 mg twice daily (group 4), nevirapine 400 mg once daily (group 5), or efavirenz 600 mg once daily (group 6). Furthermore, the tenofovir data from group 4 and group 5 were compared with the tenofovir data of group 6.

This study included data that were collected from TDM samples during 2002 for the control groups 1-3 (ie, during a period when tenofovir DF was not yet available in the Netherlands) and during 2003 and 2004 for the tenofovir DF groups. Data were collected from the application form that accompanied each TDM sample, including: gender, age, body weight, indication for TDM, concomitant medication, plasma levels of efavirenz, nevirapine or tenofovir, and time after dosing of each medication. Medication adherence data were collected by interviewing the patients. Noncompliant patients were excluded from the analysis. In instances in which there was no information on use of other antiretrovirals or if the reason for TDM was suspected noncompliance, these data were excluded. Also, if there was concomitant use of rifampin or lopinavir/ritonavir or atazanavir (with or without ritonavir), these data were excluded from the study owing to the potential confounding effects on pharmacokinetics of nonnucleoside reverse transcriptase inhibitors or tenofovir. Furthermore, only the first nevirapine, efavirenz, and tenofovir plasma level of each patient was used (to exclude bias from repeated applications), and any patient included in one of the tenofovir groups was not allowed to contribute data for the control group. Each patient took the medication for at least 2 weeks, to ensure steady-state. For the analysis of nevirapine or efavirenz plasma levels, samples of all time points were used.

For the analysis of tenofovir plasma levels, only samples at least 4 hours after medication intake were used in this study to avoid problems of interpretation during the absorption phase. Two approaches were used to compare the tenofovir plasma levels of the nevirapine groups (groups 4 and 5) with the efavirenz control group (group 6). First, tenofovir plasma levels (expressed as mg/L) of group 4 and 5 were compared with the plasma levels of group 6. Second, tenofovir concentration ratios of group 4 and 5 were compared with the concentration ratios of group 6. The concentration ratio represents the measured plasma concentration compared with the time-adjusted average concentration, as measured in a reference

population[6]. A population curve was constructed for the period 0-24 h after dose, using results of a study with tenofovir DF in 24 healthy volunteers[7]. These volunteers were given tenofovir DF 300 mg once daily over a 10-day period without any other medication. The curve was composed using the median values of the plasma concentrations for each time point.
The HIV Monitoring Foundation (SHM) follows all HIV-infected patients as a national cohort, and patients have given informed consent. This cohort protocol has been approved by all institutional review boards of the 22 Dutch treatment centers for HIV-infected patients.

Bio-analysis

Nevirapine

Nevirapine plasma concentrations were determined using a validated high-performance liquid chromatography assay with an ultraviolet detector according to a modified method[8]. An Omnispher 5 C18, 250x4.6 mm analytical column was used protected by a Chromguard RP ss 10x3 mm column (both from Varian, The Netherlands). The method involved extraction of the drug from 150 µL human plasma by adding 150 µL perchloric acid (0.55 M). Fifty µL of the clear supernatant was injected onto the column. The flow rate was 1.5 mL/min. The mobile phase consisted of a mixture of phosphate buffer (600 mM; pH 6.5)/acetonitrile (75:4; vol/vol) that resolved the drug from endogenous matrix components and other drugs that were possibly present. Chromatographic analysis was performed at ambient temperature under isocratic conditions with a wavelength of 280 nm. The retention time of nevirapine was 5.6 minutes. The concentrations of the quality controls used were 0.2, 1, and 5 mg/L. The intra- and interassay coefficients of variation were less than 4% for all quality controls. The lower limit of quantification was 0.15 mg/L. The rate of recovery of nevirapine from human plasma was 102%.

Efavirenz

Efavirenz plasma concentrations were determined according to a modified method[9]. After protein precipitation with acetonitrile, 20 µL of clear supernatant was injected into the chromatographic system. Chromatographic analysis was performed at ambient temperature with ultraviolet detection at 251 nm by using an Omnispher 5 C18, 150x4.6 mm analytical column (Varian, the Netherlands) protected by a Chromguard RP ss 10x3 mm column (Varian, The Netherlands). The flow rate was 1.0 mL/min. The mobile phase consisted of a mixture of potassium dihydrogen phosphate with acetonitrile (35%/65%; vol/vol)). The retention time of efavirenz was 4.7 minutes. The concentrations of the quality controls used were 0.4, 1.6, and 10 mg/L. The intra- and interassay coefficients of variation were less than 1% for all quality controls. The lower limit of quantification was 0.20 mg/L. The rate of recovery of efavirenz from human plasma was 106%. Both the nevirapine and efavirenz assays were externally validated

by the International Program for Quality Control of Therapeutic Drug Monitoring in HIV Therapy[10;11] and by the Quality Assurance Program for Clinical Measurement of Antiretrovirals[12].

Tenofovir

Tenofovir plasma concentrations were determined by use of a high-performance liquid chromatography assay previously described[7]. A Symmetry Shield RP18 analytical column was used (3,5 µm; 150 by 4.6 mm; Waters, Etten-Leur, the Netherlands). The method involved extraction of the drug and internal standard, adefovir (Gilead Sciences, Foster City, CA), from 100 µL human plasma by acetonitrile. After evaporation of the supernatant, 200 µL of 0.34% chloroacetaldehyde in 50 mM acetate buffer (pH 4.5) was added. Ten µL of the fluorescent compounds were injected onto the column. The flow rate was 1 mL/min. The mobile phase consisted of a mixture of phosphate buffer (50 mM, pH 6.8)/acetonitrile (96:4; vol/vol) that resolved the drug and the internal standard from endogenous matrix components and other drugs that were possibly present. Chromatographic analysis was performed at 30°C under isocratic conditions with extinction and emission wavelengths of 232 and 420 nm, respectively. The retention times of tenofovir and the internal standard, adefovir, were 6.34 and 3.90 minutes, respectively. The concentrations of the quality controls used were 0.03, 0.21, and 1.05 mg/L. The intra- and interassay coefficients of variation were less than 4% for all quality controls. The lower limit of quantification was 0.0045 mg/L. The rate of recovery of tenofovir from human plasma was 86%. The tenofovir assay was externally validated by the Quality Assurance Program for Clinical Measurement of Antiretrovirals[12].

Statistical analysis

All statistical evaluations were performed with SPSS software version 11.0 (SPSS Inc. Chicago, IL, USA). Plasma levels of nevirapine, efavirenz, tenofovir, and tenofovir concentration ratios were log transformed before statistical analysis. Geometric means were calculated for the different groups; the control groups of nevirapine and efavirenz were compared with their accessory groups with use of the 2-sided Student t-test for independent samples. The tenofovir plasma levels and tenofovir concentration ratios of group 4, 5, and 6 were compared using an one-way analysis of variance (ANOVA). The values of weight, age, and time of sampling were not transformed and were compared using the Mann-Whitney test. A *P*-value <0.05 was considered significant in all analyses.

RESULTS

Demographics

The median (range) demographic data for each group are reported in Tables 1A and 1B. Data were collected for 272 patients in the 200 mg twice daily nevirapine control group (group 1) and for 39 in the tenofovir DF + nevirapine twice daily group (group 4). In both the control and tenofovir DF groups, approximately 75% of patients were male. The 400 mg once daily nevirapine control group (group 2) was composed of 18 patients, whereas the tenofovir DF + nevirapine once daily group (group 5) included 94 patients. The distribution of males and females in these two groups was comparable to that of the other groups, including approximately 75% male subjects.

For efavirenz, data were collected for 126 patients in the efavirenz control group (group 3) and for 118 in the tenofovir DF + efavirenz group (group 6). In the control and tenofovir DF groups 81% and 73% of patients were male, respectively.

In the efavirenz + tenofovir DF control group (group 6) 70 patients were included, whereas in the nevirapine once daily + tenofovir DF group (group 5) and in the nevirapine twice daily + tenofovir DF group (group 4) 63 and 17 patients were included, respectively. The groups used for the tenofovir plasma levels and tenofovir concentration ratios (group 4, 5, and 6) were smaller than their accessory groups 1, 2, and 3, because for group 4, 5, and 6, only samples were used at least 4 hours after medication intake (Table 2). No significant differences were found between the nevirapine control groups and the tenofovir DF groups for age. For weight there was a significant difference between the patients in the 200 mg nevirapine control group (group 1) and the nevirapine 200 mg twice daily + tenofovir DF 300 mg once daily group (group 4); the weights were 76.7 kg and 69.5 kg for group 1 and 4, respectively.

Pharmacokinetic data

No significant differences were found between the nevirapine control groups and the tenofovir DF groups for time after intake of medication and nevirapine concentrations (Table 2).

For the efavirenz groups (3 and 6) a significant difference was observed between the groups with and without tenofovir DF in time after intake (Table 2), but not in efavirenz plasma levels.

Finally, no difference was found between the control group (6) and the nevirapine + tenofovir DF groups (4 and 5) regarding tenofovir plasma levels and tenofovir concentration ratios (Table 2). Only time after intake of the 200 mg nevirapine tenofovir DF group (group 4)

Table 1A: *Demographic data for all nevirapine and efavirenz groups*

		Control group (1) 200 mg NVP BID	TDF group (4) 200 mg NVP BID +300 mg TDF QD	Control group (2) 400 mg NVP QD	TDF group (5) 400 mg NVP QD + 300 mg TDF QD	Control group (3) Efavirenz 600 mg QD	TDF group (6) Efavirenz 600 mg QD + TDF 300 mg QD
N(%)	M	203 (75)	29 (74)	14 (78)	67 (71)	102 (81)	86 (73)
	F	69 (25)	10 (26)	4 (22)	27 (29)	24 (19)	32 (27)
Age(y)		41 (18-68)	44 (19-65)	38 (26-57)	41.5 (21-68)	43 (19-710)	39.5 (18-68)
Weight(kg)		76.4 (48-140)	71.0 (39-97)*	71 (52-104)	71.5 (38-115)	73 (48-112)	72 (48-106)

M=males, F=females, Data are medians (range), NVP=nevirapine, EFV=efavirenz, TDF=tenofovir disoproxil fumarate

**Significant difference p=0.01 between group 1 and 4*

Table 1B: *Demographic Data for Tenofovir DF groups*

	TDF group (4) 200 mg NVP BID + 300 mg TDF	TDF group (5) 400 mg NVP QD + 300 TDF	TDF control group (6) Efavirenz 600 mg QD + TDF 300 mg QD
N(%) M	12 (63)	45 (71)	53 (76)
F	7 (37)	18 (29)	17 (24)
Age (y)	39.0 (19-54)	40.0 (21-61)	41.5 (25-68)
Weight (kg)	70 (39-97)	72 (38-115)	75 (52-106)

M=males, F=females

Data are medians (range) NVP=nevirapine, EFV=efavirenz, TDF=tenofovir disoproxil fumarate

differed significantly from the control group 6, but this is corrected for by using concentration ratios.

All the separate plasma levels from the different groups (nevirapine, efavirenz, and tenofovir) are shown in Figures 1, 2, and 3, respectively. The tenofovir concentration ratios of the 3 tenofovir groups are presented in Figure 4.

DISCUSSION

The primary objective of this study was to investigate the effect of tenofovir DF (300 mg once daily) on the plasma nevirapine (200 mg twice daily or 400 mg once daily) levels. This retrospective study demonstrated that coadministration of tenofovir DF with nevirapine (200 mg twice daily or 400 mg once daily) in a large number of HIV-infected patients had no effect on nevirapine plasma levels compared with administration of nevirapine alone.

The secondary objective of this study was to explore the effect of nevirapine on tenofovir plasma levels. This was studied by comparing tenofovir plasma levels and tenofovir concentration ratios from patients using nevirapine (200 mg twice daily or 400 mg once daily) and tenofovir DF (300 mg once daily) with the levels in patients using efavirenz (600 mg once daily) and tenofovir DF. The results of this study showed that nevirapine, whether administered once or twice daily, had no significant effect on the tenofovir plasma levels or concentration ratios.

Furthermore, the effect of tenofovir DF on efavirenz pharmacokinetics (and vice versa) was assessed and no effects were found.

Table 2: *Pharmacokinetic Data*

	Control group (1) 200 mg NVP BID 300 mg	TDF group (4) 200 mg NVP BID + 300 mg TDF QD		Control group (2) 400 mg NVP	TDF group (5) 400 mg NVP + 300 mg TDF QD		Control group (3) EFV 600 mg QD	TDF group (6) EFV 600 mg + TDF 300 mg QD	
ARV	NVP	NVP	TFV	NVP	NVP	TFV	EFV	EFV	TFV
N	272	39	19	18	94	63	118	126	70
Time (h)[a]	5.2	5.2	9.0*	10.1	11.0	16.0	13	14.2*	14.9
	(0.3-14.0)	(0.2-14.3)	(4-24)	(0.3-24.0)	(0.0-24.4)	(4.6-24.4)	(0.5-21.5)	(0.5-23.9)	(5.5-23.9)
EFV (mg/l)							2.34	2.22 (<0.15-11.4)	
Range							(0.82-16.5)		
NVP (mg/l)	5.68	6.48		5.25	4.85				
Range	(0.7-22.5)	(3-16.9)		(2.40-9.50)	(<0.15-20.2)				
TFV (mg/l)			0.082			0.068			0.078
Range			(0.029-0.222)			(<0.0045-0.205)			(<0.0045-0.537)
TFV Ratio			0.57			0.53			0.59
Range			(0.22-0.78)			(0.02-1.56)			(0.02-4.40)

Data are geometric means (range), except for Time: median (range), BID= twice daily, QD= once daily, ARV= antiretroviral drug tested, N=number of patients, NVP=nevirapine, TDF= tenofovir disoproxil fumarate, EFV=efavirenz, TFV= tenofovir, TFV Ratio= tenofovir concentration ratio (concentration ratio represents the measured plasma concentration compared with the time-adjusted average concentration, as measured in a reference population)

For NVP QD group 4 is compared to group1, for NVP BID, group 5 is compared to group 2, for EFV group 6 is compared to group 3, and for TFV and TFV ratio, group 4 and group 5 are compared to group 6. [a]Time after last intake of medication

** Significant difference p=0.001 between group 4 and group 6.*

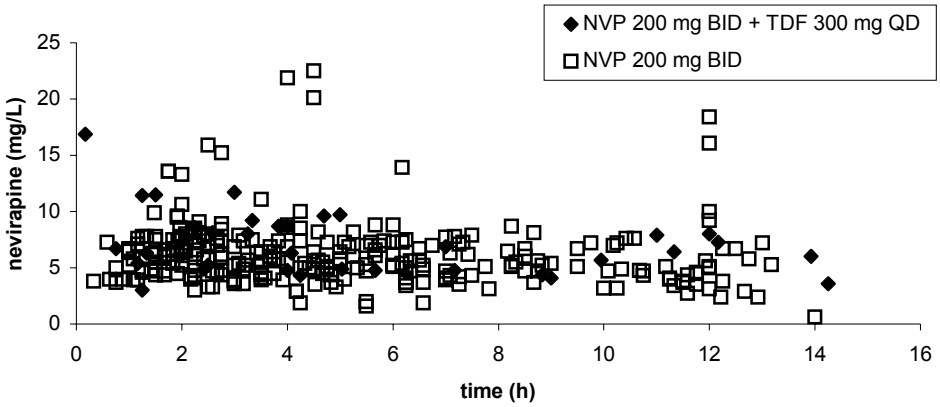

Figure 1A: *Nevirapine twice daily plasma levels*

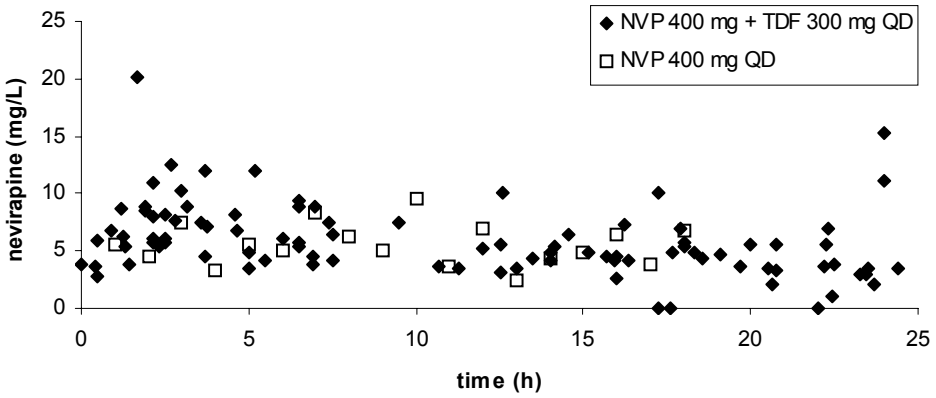

Figure 1B: *Nevirapine once daily plasma levels*

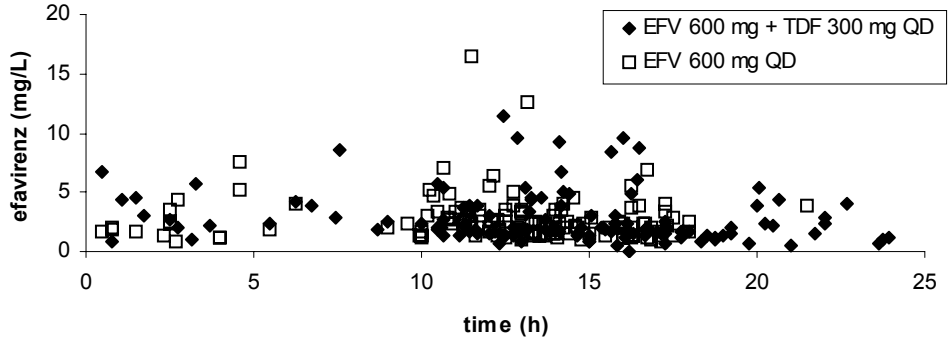

Figure 2: *Efavirenz plasma levels*

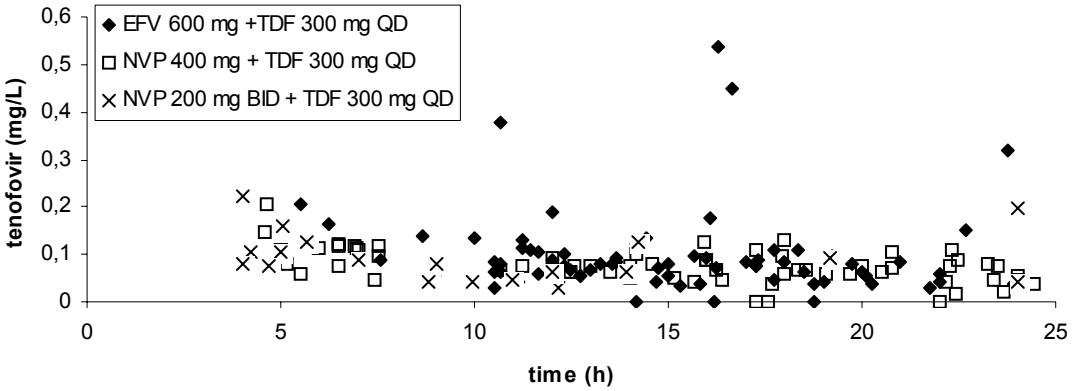

Figure 3: *Tenofovir plasma levels*

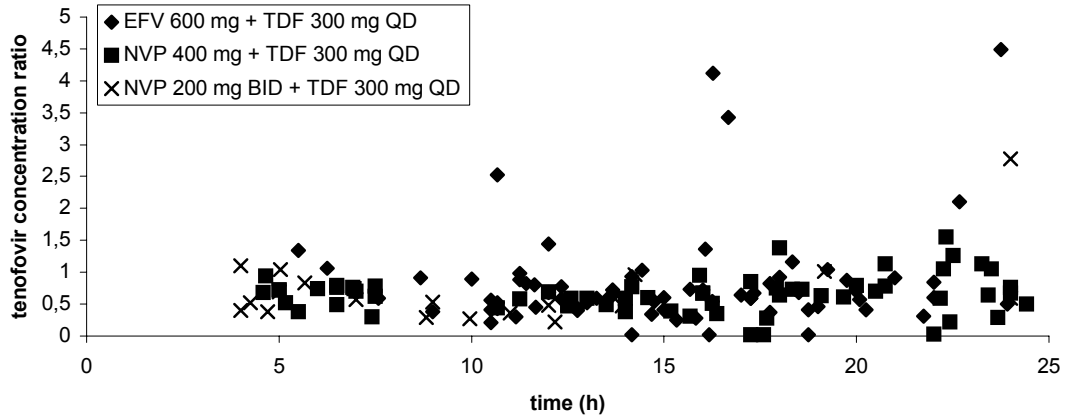

Figure 4: *Tenofovir concentration ratios*

The efavirenz data from the efavirenz groups are consistent with the results from a crossover drug-drug interaction study conducted in healthy volunteers[5]. As such, results from this study demonstrated the potential usefulness of using a TDM database to assess possible drug-drug interactions in HIV-infected patients.

The sometimes large differences in sample size in the different study groups are the result of the retrospective nature of this study, and inherent to TDM database analyses.

Although crossover drug-drug interaction studies allow for the most precise assessment of pharmacokinetics and detection of small, clinically insignificant drug-drug interactions, appropriate use of clinical data, including TDM results, offer an additional useful tool for assessing drug-drug interactions. This is of particular importance when pharmacokinetic studies in healthy volunteers may not be appropriate, eg, for a drug like nevirapine in which an

increased risk of serious liver toxicity in subjects with higher CD4 cell counts has been observed[13].

In this study plasma concentrations of tenofovir were determined during its elimination phase, at least 4 hours after intake. In routine clinical care it is often difficult to draw a blood sample at a strictly defined timepoint and because the data of this study were collected from routine TDM samples, the samples were drawn at different times after intake; therefore, tenofovir concentration ratios were used for comparison across groups to correct for time after intake. Additionally, for nevirapine all time points during dosing intervals were used because time after intake is not relevant owing to the long half-life of this agent[14]. For efavirenz it is known that the half-life of efavirenz is 40-55 hour, and therefore, we decided to use samples of all timepoints during the dose interval[15].

Because tenofovir is a prodrug that requires intracellular activation to an active diphosphate derivate, and a relationship between the plasma tenofovir levels and intracellular derivate has not been established yet, measuring the intracellular tenofovir diphosphate levels might be more useful than measuring plasma levels. However, measurement of intracellular levels of tenofovir diphosphate is technically difficult, time consuming and not widely available. For that reason measuring plasma levels of tenofovir is the alternative.

Furthermore, no data were available for patients who were using only tenofovir DF, therefore plasma concentrations of tenofovir and tenofovir concentration ratios from patients to whom nevirapine was coadministered were compared with plasma concentrations of tenofovir and tenofovir concentration ratios in the efavirenz group.

The mean tenofovir concentration ratios of the 3 tenofovir DF groups 4, 5, and 6 were 0.57, 0.53, and 0.59, respectively (Table 2) compared with the reference population[7]. These results suggest that tenofovir concentrations in this study are lower than the tenofovir concentrations in the reference population. The pharmacokinetics of tenofovir has been evaluated in healthy volunteers and HIV-infected individuals. Tenofovir pharmacokinetics have been found to be similar between these populations[2].

However, several reasons can explain the difference between the tenofovir results from this study and the reference population. First, a high fat meal (700-1000 kcal) can have an effect, as coadministration with food increases the absorption[2]. The volunteers in the reference population took their tenofovir DF with a standardized breakfast of 550 kcal[7], whereas for the HIV-infected patients of this study, this information is not known, because tenofovir DF may be taken with or without food[2]. Second, the medication intake of the healthy volunteers was monitored and the time was recorded carefully, as was the time of blood drawing. The patients in this study took their medications at home and the time was recorded during their visit at the hospital and therefore probably subjected to more variation.

Although a small but significant difference was found between the body weights of the 200 mg nevirapine control group and the tenofovir DF group, it is unlikely that this finding has influenced the results as body weight is not associated with plasma nevirapine concentrations[16].

In conclusion, the data from this study demonstrate that the addition of tenofovir DF to efavirenz or nevirapine in HIV-infected patients does not influence the plasma levels of nevirapine or efavirenz. Furthermore, neither efavirenz nor nevirapine have any effect on the plasma levels of tenofovir. This suggests that these drugs can be coadministered without the need for dose adjustments.

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CHAPTER 9

Nevirapine Plasma Concentrations are Still Detectable After More Than Two Weeks in the Majority of Women Receiving Single-Dose Nevirapine
Implications for Intervention Studies

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ABSTRACT

Background: Single-dose nevirapine is a highly cost-effective strategy to reduce perinatal HIV-1 transmission. Its major disadvantage is the selection of nevirapine resistance in 20% to 30% of women, probably attributable to the long elimination half-life of nevirapine. To develop intervention strategies, it is important to know the interpatient variability in nevirapine half-life in women receiving a single dose of nevirapine

Methods: HIV-negative, healthy, nonpregnant Dutch women were eligible for this study. After administration of a single 200 mg dose of nevirapine to the subjects, blood was sampled for measurement of nevirapine twice a week for a total of 21 days. Nevirapine plasma levels were determined by a validated high-performance liquid chromatography method with a lower limit of quantification of 0.15 mg/L. The primary endpoint was the first sample with an undetectable nevirapine concentration.

Results: Forty-four subjects participated. The median age, height and body weight (interquartile range) were 26 (21-33) years, 1.72 (1.68-1.75) m, and 64 (59-75) kg, respectively. The median elimination half-life of nevirapine was 56.7 hours with a range of 25.6 to 164 hours. The time to the first undetectable nevirapine plasma concentration was 10 days in 4 subjects, 14 days in 12 subjects, 17 days in 12 subjects, and 21 days in 9 subjects. In the remaining 7 subjects, nevirapine was still detectable on day 21, the last day of sampling. Time to an undetectable nevirapine plasma concentration was influenced by oral contraceptive use but not by age, height, body weight, body surface area, alcohol use, or smoking.

Conclusions: Most women who received a single 200 mg nevirapine dose still had detectable plasma concentrations of nevirapine after more than 2 weeks. This information is valuable for designing intervention studies to prevent the development of nevirapine resistance.

INTRODUCTION

Without the use of preventive measures, the risk of mother-to-child transmission (MTCT) of HIV-1 is estimated to vary between 25% and 48%. Several preventive strategies have been evaluated, but most of them are too expensive to implement in resource-limited countries. The regimen of a single dose of nevirapine to the mother just before delivery and a single dose of nevirapine to the newborn between 24 to 72 hours after birth reduces the risk of MTCT by 50% and is affordable in many situations[1].

Recent studies, however, have shown that this single dose to the mother can induce nevirapine resistance in 20% to 30% of the mothers[2]. The development of this resistance may have major implications. First, it is uncertain whether a subsequent course of nevirapine is still effective for the prevention of MTCT when these women become pregnant again. Second, the efficacy of subsequent treatment with nevirapine-based highly active antiretroviral therapy (HAART) regimen will be diminished when the patient is harboring a resistant virus[3]. Finally, nevirapine-resistant strains may be transmitted to other people.

The mechanism of the occurrence of nevirapine resistance after a single dose is most likely related to the long elimination half-life of nevirapine and the low genetic barrier to resistance. Small pharmacokinetic studies have demonstrated that the elimination half-life after a single dose of nevirapine is approximately 60 hours[4]. This implies that plasma concentrations of nevirapine are detectable in mothers for several days after delivery. The subtherapeutic but detectable plasma levels present the perfect environment for the occurrence of resistance, as the concentrations may be subinhibitory for several days.

The primary objective of this study was to investigate the intersubject variability in and potential influencing factors of the decay of plasma nevirapine concentrations after a single 200 mg dose. A secondary objective was the evaluation of the use of saliva as an alternative to blood sampling for measurement of nevirapine concentrations. The study was conducted in the Netherlands as a prelude to a similar one in Tanzania.

METHODS

The present study was a single-centre, open-label, single-dose, single-period pharmacokinetic study. Nonpregnant healthy women aged 18 to 40 years were eligible for enrollment after pre-entry and laboratory evaluation. Women who tested positive for HIV and/or hepatitis B or C virus were excluded. The study protocol was reviewed and approved by Ethics Committee of the Radboud University Medical Centre Nijmegen, The Netherlands. Informed consent was obtained from all women prior to enrollment.

All study subjects received a single oral dose of 200 mg of nevirapine on day 0, and the Principal Investigator directly observed medication ingestion. Sampling of blood and saliva was done just before and 3, 7, 10, 14, 17, and 21 days after a single 200 mg dose of nevirapine. Stimulated saliva was obtained by a salivette (Sarstedt, Etten-Leur, the Netherlands) using a dental cotton roll impregnated with citric acid (20 mg), which stimulates the salivary flow. Study subjects were asked to chew on the cotton roll for approximately 1 minute. Saliva was obtained by centrifugation of the cotton roll. The plasma and saliva samples were stored at – 40°C until analysis. Plasma and saliva concentrations of nevirapine were determined by a validated high-performance liquid chromatography assay with ultraviolet detection[5]. The lower and upper limits of quantification were 0.15 and 15 mg/L, respectively. The intra- and interday precision ranged from 1.3% to 3.9% and from 1.9% to 3.0%, respectively. The accuracy of the assay ranged from 91.5% to 102.6%.

The typical median inhibitory concentration (IC_{50}) value of nevirapine is 0.1 mg/L; corrected for 60% protein binding, this corresponds to a plasma level of approximately 0.2 mg/L. It is currently unknown, however, at what plasma level nevirapine selects for resistance. Clinical studies have determined that effective concentrations of nevirapine are greater than 3 to 4 mg/L, while levels less than 0.1 to 0.2 mg/L do not have selective pressure. Therefore, any plasma level between 0.2 and 3.0 mg/L has been defined by us as subtherapeutic. The following patient factors were tested for an association with the time to undetectable nevirapine plasma concentration: age, height, weight, body surface area, alcohol use, smoking habits, and oral contraceptive use.

RESULTS

Forty-four non-pregnant healthy women were enrolled in the protocol. The median age, height, and body weight (interquartile range) were 26 (21-33) years, 1.72 (1.68-1.75) m, and 64 (59-75) kg, respectively. Other than 1 Asian woman and 1 woman of mixed background, the remaining 42 women were Caucasian.

The pharmacokinetic parameters of the study subjects are presented in Table 1. The median elimination half-life ($t_{1/2}$) for nevirapine in plasma was 56.7 hours with a range of 25.6 to 164 hours. Maximum nevirapine plasma levels at day 3 (first post-dose measurement) ranged from 0.36 to 1.59 mg/L with a median value of 0.71 mg/L. The median time to the first undetectable nevirapine plasma concentration was 17 days. There were 7 subjects in whom nevirapine was still detectable on day 21, the last day of sampling (Fig. 1).

Except for oral contraceptive use, none of the other patient characteristics seemed to be related to the time to an undetectable nevirapine concentration in plasma. There were 17 women who reported taking oral contraceptives, and they had a median time to the first

Table 1: *Pharmacokinetic parameters of nevirapine after a 200 mg single oral dose (median values + range)*

	Plasma	Saliva
CL/F (L/h.kg)	0.04 (0.02 – 0.10)	
T-half (h)	56.7 (25.6 - 164.1)	77.1 (35.8-264.7)
Vd/F (L/kg)	2.8 (0.8 - 5.3)	
C_{max} (mg/L)	0.71 (0.36 - 1.59)	0.35 (0.03-0.77)
Time to undetectable concentration (days)	17 (10 - >21)	14 (7-21)

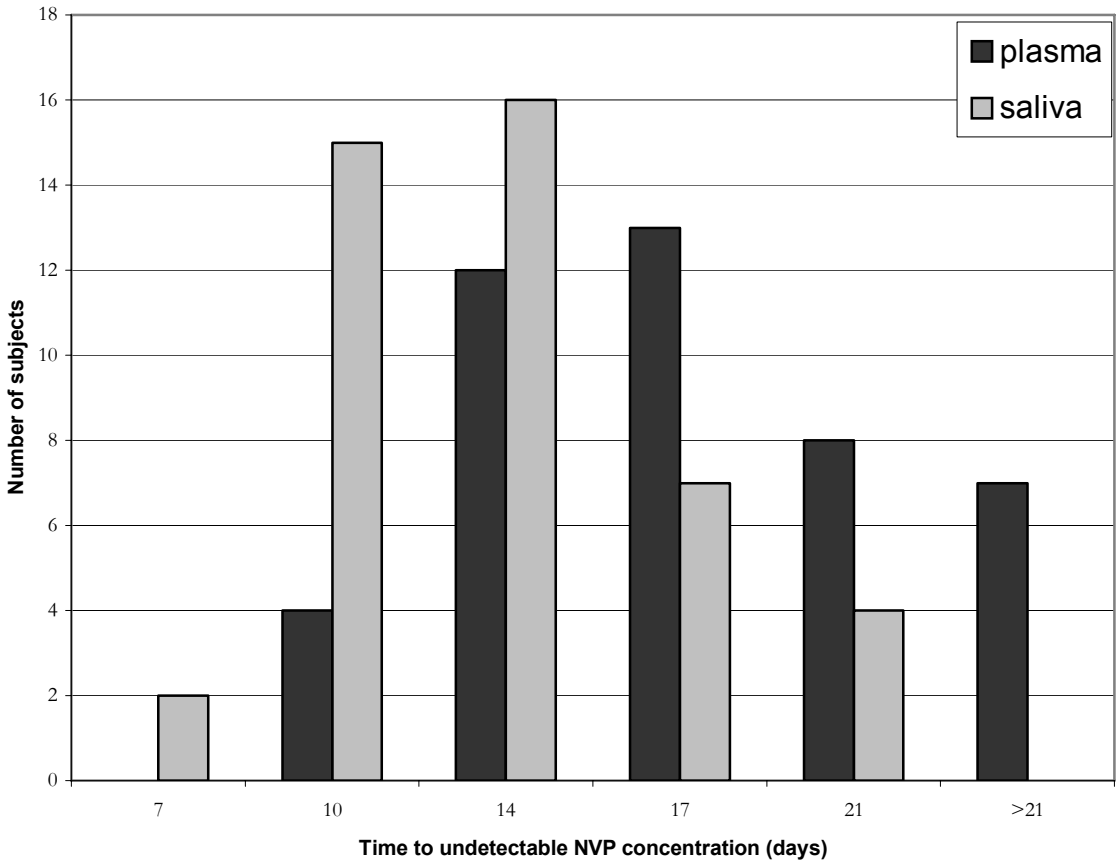

Figure 1: *Distribution of time to undetectable NVP concentration (in days) for the 44 included subjects*

undetectable nevirapine plasma level of 21 days. This was significantly longer than for the remaining 27 women who reported not taking oral contraceptives (14 days; P < 0.001). The difference in median plasma half-life of nevirapine in oral contraceptive users versus nonusers was not significant (69.7 versus. 52.8 hours), respectively (p=0.053).

Saliva nevirapine concentrations were approximately half of the values observed in plasma. Nevirapine levels in saliva were significantly correlated with nevirapine levels in plasma at the first day of sampling: $[NVP]_{saliva} = -0.002 + 0.495 \times [NVP]_{plasma}$ (R^2=0.531, F = 47.496, p < 0.001)**.** Time to an undetectable nevirapine concentration was shorter in saliva than in plasma: median values were 14 and 17 days, respectively (Fig. 1).

DISCUSSION

In this study of 44 healthy, non-pregnant, HIV-1-uninfected Dutch women, a single dose of 200 mg of nevirapine had an average half-life of 56.7 hours (or 52.8 hours women who did not use contraceptives). Nevirapine levels remained detectable in plasma for a median of 17 days (range: 10 to >21 days). In 16% of women, nevirapine was detectable at the last measured time point, 21 days after the single dose. Thus, a single dose of nevirapine was associated with persistent measurable drug levels beyond 3 weeks after administration.

It is clear that our study population of healthy non-pregnant Dutch women of childbearing age is not similar to the setting in Tanzania (or other sub-Saharan African countries), where HIV-infected pregnant women are black and have different dietary habits, body weights, and co-medication, for example. Nevertheless, the median nevirapine half-life that we observed in our group of 44 subjects (56.7 hours) is not very different from the average value as reported by Musoke et al[4] in a smaller group of pregnant HIV-infected Ugandan women receiving single-dose nevirapine (61.3 hours). We were not able to identify any significant patient factor (other than oral contraceptive use) that was associated with an influence on nevirapine half-life. Oral contraceptives may be able to inhibit hepatic metabolism of nevirapine, although this effect was previously not observed (and thus not expected by us) in a formal drug-drug interaction study[6]. It must be noted, however, that our study was not designed to address causality between oral contraceptive use and nevirapine half-life.

Most importantly, our data describe the window of opportunity for the virus to select for nevirapine resistant mutations. The longer the time to an undetectable nevirapine level in plasma, the longer the virus has time to replicate. In 2000, Jackson et al[7] reported that among the 15 women in the HIVNET 006 trial in whom virus was tested for the K103N mutation, the 3 women who developed the mutation had a significantly longer elimination half-life of nevirapine than the 12 women in whom no resistance was detected (74.8 versus

51.8hours; p=0.01). Thus, one of the most rational interventions is the addition of other antiretroviral agents after delivery to cover this window of opportunity for the virus to select for nevirapine resistance. Recently, preliminary data were presented that short courses (4-7 days) of zidovudine+lamivudine (Combivir®) added to single-dose nevirapine in the prevention of MTCT significantly reduced the development of nevirapine resistance when compared to no intervention[8]. One could speculate that this will not be sufficient to prevent the development of all nevirapine mutations. Indeed, nevirapine resistance was not fully absent in the intervention arms. Extending the duration of administering additional antiretroviral agents after delivery may also increase the development of resistance to these drugs.

It may be attractive to use alternative methods, other than collecting blood samples for measurement of exposure to nevirapine. As reported earlier, nevirapine can be detected in saliva[9]. In our study, we observed a very strong correlation between nevirapine levels in saliva versus plasma, and in almost al subjects an undetectable nevirapine level was detected 3 days earlier in saliva than in plasma (see Fig. 1). Collecting saliva samples for measurement of nevirapine levels has the advantage of taking samples at home (by the patient herself; with no skilled personnel needed), less discomfort for the patient, and less infection risk for health care workers who draw the sample.

In conclusion, most women who received a single nevirapine dose of 200 mg still had detectable plasma concentrations of nevirapine after more than 2 weeks. This information is valuable for designing intervention studies to prevent the development of nevirapine resistance.

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PART V

GENERAL DISCUSSION

INTRODUCTION

This thesis intended to make a contribution to therapeutic drug monitoring (TDM) of HIV treatment and bridging the laboratory and clinic. TDM is employed to measure and follow blood drug levels so that the most effective dosage can be determined, for each individual patient, with toxicity prevented. TDM is also utilized to identify noncompliant patients.
Many different factors influence blood drug levels such as analytical techniques, patient factors, and drug factors. For analytical techniques it is important to know the quality of the assay. The patient factors include age, weight and co-morbidity. Also patient compliance is a key factor. For drug factors, the route of administration of the drug, the drug's absorption, excretion, delivery rate, drug dosage, and co-medications should be taken into consideration during TDM.
The studies described in this thesis have explored the following aspects of TDM: analysis of protease inhibitors in plasma and the stability of protease inhibitors in plasma, cross reaction of efavirenz on drug screening assay, quality of TDM services, drug-drug interactions, and drug levels of single-dose nevirapine.

BIO-ANALYSIS OF ANTIRETROVIRAL DRUGS AND QUALITY OF TDM SERVICES

Bio-analysis of antiretroviral drugs in plasma is a requirement for TDM. Since the introduction of the protease inhibitors in 1995, methods have been developed for the analysis of single protease inhibitors and for simultaneous determination of several protease inhibitors[1]. In *chapter 1* a bio-analytical method for the simultaneous determination of 6 protease inhibitors and nevirapine is described. The lower limit of quantification for the several antiretroviral drugs is low enough for quantitation of concentrations below trough concentrations of the single protease inhibitors or below presumed therapeutic thresholds for protease inhibitors.
This is in contrast with the results of three participants in the international interlaboratory quality control program described in *chapter 4.* These participants reported to have their lower limits of quantification above low or trough concentrations of amprenavir, indinavir, and ritonavir. This quality control program also showed that lower concentrations are more difficult to assess accurately than medium or higher plasma concentrations. Furthermore, the program revealed that about 20% of the results reported were outside the acceptable limits of 80% to 120% accuracy and only 3 out of 30 laboratories were able to report all results within the acceptable limits.
As a result, the possible negative impact of the analysis on TDM and pharmacokinetic studies is probably larger than expected because dose adjustments and switching therapy

might occur based on inaccurate information about concentrations and pharmacokinetic studies might produce incorrect results.

The validation of bio-analytical high-performance liquid chromatography (HPLC) methods has been described by the International Conference on Harmonization[2]. The intent of bio-analytical HPLC methods validation is to demonstrate that the method is suitable for its intended purpose, accurate quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum or urine. According to the guidelines this validation includes determination of selectivity, accuracy, precision, recovery, stability, lower limit of quantification, and calibration/standard curve concentration-response. One important item is not included in the guidelines: participating in an external quality control program if available for the analyte tested. When an external quality control program is part of the validation of a new method, laboratories are alerted to undetected analytical errors and are able to improve their assays

As described in *chapter 4,* an external quality control program is helpful to detect possible problems with the analytical method. The reasons for unsatisfactory measurements were reported. There was an explanation for 86% of all the unsatisfactory results. The majority of the reasons were of technical origin, varying from dilution error, acceptance of an analytical run even though controls were out of range, to aging stock solutions, and stock solutions made of impure substances.

Papers reporting new analytical methods should include successful results of participating in a quality control program to assure acceptable accuracy of the reported method. For example the recently published methods for the determination of nucleoside analogue reverse transcriptase inhibitors[3] and the determination of protease inhibitors[4] by HPLC reported satisfactory results obtained from a quality control program and for that reason these methods are suitable for TDM as long as participation in a quality control program is continued successfully. Furthermore, clinicians should make sure that the laboratory, to which their TDM samples are sent, participates in an extern quality control program at least twice a year successfully. In addition, papers that describe results from pharmacokinetic studies and trials in which antiretrovirals are determined in various centers, should also include results from external quality control programs, to assure the reliability of the results. Recently, several papers are published containing this information[5-8]. This is important because the results of these kinds of studies can have great impact on the treatment of HIV-infected patients. The importance of an accurate method for quantification of antiretroviral drugs should not be underestimated.

In addition when using another matrix than the one the assay is developed for, a (partly) revalidation is needed. The recovery from the matrix, the stability, the intraday and interday variation and interfering substances might be different. Most HPLC methods described are

developed for measurements in plasma. There is no quality control program available for the measurement of any drug in saliva, or seminal plasma. Nevertheless, when reporting data of drugs in other matrices than plasma, validation results should be added. Published data of indinavir in cerebrospinal fluid (CSF) and in seminal plasma[9], zidovudine and lamivudine in seminal plasma[10], lopinavir and indinavir in CSF and seminal plasma[11] only contain a reference of an HPLC method in blood plasma. On the other hand Solas et al[12] measured protease inhibitors in seminal plasma and CSF and reported how they performed the analysis together with the validation parameters. This should be required before a paper can be accepted by a scientific journal.

In *chapter 6* data of lopinavir in seminal plasma are presented without a complete set of validation data of the analysis. Only the intra- and interday variations are presented, although a validation needed for another matrix was performed. In fact these data should have been added to the article in *chapter 6***.**

Furthermore, long-term stability of the antiretroviral agents in plasma has to be known, especially for retrospective pharmacokinetic studies. As described in *chapter 2* almost all protease inhibitors currently available are stable for at least 18 months at –20^{o}C. Before a pharmacokinetic retrospective study is conducted, stability of the drug studied should be determined. According to the guidelines for bio-analytical method validation[2], long-term stability should be tested by evaluating at least three aliquots of each low and high control samples after a storage time that exceed the time between the date of first sample collection and the date of last sample analysis. These control samples usually contain an organic solvent in which the drug is dissolved. Patient samples on the other hand do not contain organic solvents, but contain possible metabolites and comedication. To be really sure of the stability, it might be better to use plasma samples of HIV-infected patients to assess the long-term stability, instead of artificial quality control samples. Anyhow, stability should be tested for a long period. During validation of a new developed method, long-term stability is usually tested for 2 to 6 months at the most (*chapter 2)*, while for example the 2NN study[13] collected efavirenz and nevirapine samples during 2 years (2000 and 2001) and analyzed them in 2004 (personal communication, B. Kappelhoff) without any comment on the stability of these agents. The results of this study might be influenced by the possible degradation of efavirenz or nevirapine. Therefore, results of retrospective pharmacokinetic studies should always be accompanied by stability tests for at least the duration of storage, preferably in HIV-infected patient plasma samples.

Besides the quality of the analyses, the interpretation of the plasma levels as part of TDM is of importance. The results of the patient cases that were part of the interlaboratory quality control program revealed to be variable among the respondents and the recommendations

given were partly incomparable (*chapter 5*). The drug levels were easy to judge, as ± 90% were able to correctly do so. Therapeutic ranges of the protease inhibitors and nonnucleoside reverse transcriptase inhibitors in plasma are commonly known and published before[14;15].

On the other hand *chapter 5* showed a large variability among the respondents in their ability to give satisfactory recommendations concerning dose adjustments, changes to the regimen and drug-drug interactions. The variation in recommendations may result in implications for the patient.

PHARMACOKINETICS

Pharmacokinetics is the study of the time course of a drug in the body after administration. After dosing, absorption, distribution, metabolism, and excretion of the drugs in the body will occur. Biological, physiological, and physicochemical factors may influence the transfer processes of drugs. Because of comorbidity (tuberculosis (TB), hepatitis etc.) HIV-infected patients may need other drugs that might interact with the antiretroviral drugs.

HIV-infected patients usually receive a wide variety of drugs in addition to the antiretroviral drug regimen. Every year new data on antiretroviral drug interactions are published or presented in scientific meetings and there are numerous review articles[16-19] and websites listing all possible drug-drug interactions with antiretroviral agents.

Sheehan et al[20] have evaluated several antiretroviral drug interaction sites for quality, comprehensiveness, and functionality, and are planning to conduct this review on a regular basis. However, the management of HIV is growing in complexity as new antiretroviral agents are developed with accompanying possible drug-drug interactions. So complete data will hardly be available in such review articles.

In *chapter 7* tenofovir DF is studied in combination with the first-line tuberculostatic agent rifampin in healthy subjects. About one third of all the people infected with HIV are also infected with *Mycobacterium tuberculosis* (TB). The majority of persons co-infected with HIV and TB reside in poor countries with most cases occurring in sub-Saharan Africa and South and South-East Asia[21-23]. Although highly effective therapy exists for both HIV and TB, concomitant administration is fraught with difficulties. Problems arise when giving these two therapies concomitantly and sometimes HIV therapy is delayed to minimize the risk of drug-drug interactions and toxic effects. It is known that patients with low CD4 cell count need HIV-therapy as well as TB-therapy. To enlarge the choice of antiretroviral agents that can be combined with rifampin without risks, the combination of tenofovir DF and rifampin was studied.

Coadministration of tenofovir DF and rifampin did not result in changes in the values of the tenofovir pharmacokinetic parameters and the values of the rifampin pharmacokinetic parameters were comparable to those found in the literature. Therefore, tenofovir DF and rifampin can be coadministered without dose modifications of either one of the drugs.

Although no interaction between tenofovir DF and both efavirenz and nevirapine was expected, it could not be excluded. Tenofovir has been found to increase plasma exposure of didanosine and decreases atazanavir plasma levels both unexpectedly[24]. Furthermore, all other currently available anti-HIV agents were tested already in combination with tenofovir DF[24] except for nevirapine. For the completeness tenofovir DF combined with nevirapine had to be studied. In *chapter 8* a study is described in which tenofovir DF was studied in HIV-infected patients when combined with nevirapine and efavirenz versus efavirenz or nevirapine alone. This study demonstrated that coadministration of tenofovir DF with nevirapine or efavirenz in a large number of HIV-infected patients had no effect on nevirapine or efavirenz plasma levels compared to administration of nevirapine alone.
In contrast with the study in *chapter 7* where tenofovir DF was studied in healthy subjects, the study in *chapter 8* was conducted in a TDM database of HIV-infected subjects. Both approaches have their advantages. A study with HIV-infected patients will have the disadvantage of a heterogeneous group, high variation and possible toxicity. On the other hand, HIV-infected patients are the target group for whom the drugs are meant and HIV-infected patients may have another metabolism[25] than healthy subjects have. On the contrary, a study with healthy subjects warrants a homogeneous group, the correct number of subjects based on statistical calculations to be able to draw conclusions, sampling time after drug intake is the same for the complete group of subjects, and complete pharmacokinetic curves can be obtained.
Nevertheless, for studying the possible interaction between tenofovir DF and nevirapine in *chapter 8,* HIV-infected patients in a TDM database were chosen because giving nevirapine to healthy volunteers might be a problem. Subjects with higher CD4 cell counts have an increased risk of serious liver toxicity[26]. This database study with HIV-infected patients proves that this approach is also a useful mechanism.

In recent years, the use of nevirapine has attracted considerable attention because of its efficacy in clinical trials in reducing mother to child transmission (MTCT).
The first trial in which nevirapine was used for prevention of MTCT was the HIVNET012 trial in 1999[27]. Since then various studies with nevirapine have been performed demonstrating that nevirapine is a simple inexpensive prophylaxis, which can dramatically reduce the risk of MTCT. However, recent studies have shown development of nevirapine resistance[28].

In *Chapter 9* a study is described in which a single-dose nevirapine is given to healthy female subjects. The time to an undetectable nevirapine plasma level varied between 10 and more than 21 days. Another study in HIV-infected Thai women[5] was performed, which confirmed our data. They also found significant nevirapine concentrations for up to 20 days.

The concentration to give 50% inhibition (IC50) for nevirapine is approximately 10 ng/mL, which is approximately 400-500 times lower than the plasma concentration achieved with 200 mg twice daily nevirapine. In treatment of HIV-infected patients, 200 mg of nevirapine twice daily is the recommended dose; this dose was found to be the maximal effective dose with acceptable toxicity[29]. The steady-state concentrations achieved with this dosing regimen are 4.5 ± 1.9 mg/L[29].

However, the women who are given single dose nevirapine to reduce the risk of MTCT do not reach steady-state nevirapine levels. The plasma concentration needed to prevent HIV-transmission and to develop resistance is not known.

Furthermore, during the HIVNET012 trial with single dose nevirapine, only 200 mg nevirapine was tested. This was probably the most practical choice, as nevirapine tablets contain 200 mg nevirapine.

The lack of data (plasma concentration needed to prevent MTCT and lowest plasma at which resistance development may occur) raises the question why a lower single dose of nevirapine was never tested. A lower dose will lead to undetectable plasma nevirapine levels more rapidly than single dose 200 mg nevirapine will, and might lead to less resistance development, while it might be as effective as single dose 200 mg nevirapine is.

Nevertheless, while work must continue to identify more efficacious and safe regimens to prevent MTCT, preventing women from becoming HIV-infected should remain an important issue.

FINAL THOUGHTS

The studies in this thesis aimed to make a contribution to TDM of HIV treatment by bridging the laboratory with the clinic.

Initially, the quality control program of antiretroviral drugs in plasma should be continued and all laboratories measuring antiretroviral drugs for TDM or pharmacokinetic studies should participate in this or a similar program on a regular basis. This way the quality of the analysis is guarded. Furthermore, new approved antiretroviral drugs, which are determined in plasma, should be included in the quality control program. The quality of the determination of the antiretroviral drugs is an important starting point for TDM.

Next, advises concerning treatment of HIV-infected patients are an important part of TDM also and should be given by well-trained personnel. As reported in *chapter 5,* levels of knowledge regarding HIV treatment appeared to be variable which could have consequences for the patients. Therefore, the quality control program should include patient cases on a regular basis so health care professionals in charge of treatment of HIV-patients can test their knowledge.
In addition, new antiretroviral drugs should be tested for possible interactions with other antiretroviral drugs and other possible comedication because interactions although maybe not expected can never be excluded.

Finally, to achieve maximum information and quality of care for the (HIV)-patients, TDM should be a close collaboration between laboratory and clinic.

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Summary

This thesis intended to make a contribution to therapeutic drug monitoring (TDM) of HIV treatment and bridging the laboratory and clinic.
In the introduction (*Part I*) of this thesis the main groups of antiretroviral drugs are described and the role of TDM in the management of HIV is outlined. Furthermore, the various chapters of this thesis are introduced.

In *Part II* topics related to chemical analysis of antiretroviral drugs are presented. A requirement for TDM is the plasma level of the antiretroviral drugs. In *chapter 1* a high-performance liquid chromatographic method for the simultaneous determination of protease inhibitors and nevirapine in human plasma is described. This method is a simple accurate and precise assay and this analysis can be applied for pharmacokinetic studies and for TDM of HIV-infected patients.
In *chapter 2* the long-term stability of protease inhibitors in human plasma at different temperatures was studied. After 18 months of storage in the freezer concentrations found varied between 95% and 108% of the initial concentration, so all protease inhibitors studied were stable at -20°C and at -80°C for at least 18 months. In order to produce accurate results when analyzing plasma samples for pharmacokinetic studies, it is important to know that these samples are stable in the freezer.
In *Chapter 3* the false positive results for efavirenz and rifampin in a urine drug-screening assay are described. Clinicians, technicians, and researchers should be aware of the possibility of false positive results.

Part III focused on the quality of TDM services. Because plasma concentrations of antiretroviral drugs are an essential starting point for TDM, these concentrations should be analyzed accurately. In *chapter 4* the results of the third round of the international interlaboratory quality control program are described, in which participating laboratories were asked to measure protease inhibitors and nonnucleoside reverse transcriptase inhibitors in human plasma. This program revealed a large variability of laboratories to measure antiretroviral drugs accurately. Only 3 out 30 laboratories performed all their measurements within the accuracy limits and of all the measurements about 20% were without the acceptable ranges. The low concentrations were more difficult to measure accurately than the medium and high concentrations. Sources of error were inquired and the most errors were technical problems. The results of this study may have important implications for TDM and pharmacokinetic studies.

In *chapter 5* the results of a patient case that was also part of the third round of the international interlaboratory quality control program are presented. The case was composed of different topics related to TDM and the participants were asked to give their recommendations. Of the 30 participants in the program, only 16 returned their recommendations. The drug level was judged correctly by about 90% of the participants. On the other hand, only half of the recommendations given were satisfactory. Therefore, the quality program is not only a tool to alert laboratories to undetected analytical problems, but is also useful to realize that recommendations regarding dose adjustment, changing of a regimen, or concomitant medication, cannot be given without proper knowledge.

In *Part IV* of this thesis pharmacokinetic studies are presented. In *chapter 6* the penetration of lopinavir in seminal plasma was studied. Lopinavir had a poor penetration in the seminal plasma, as the median ratio (N=14) of the concentrations of lopinavir in seminal plasma and in blood plasma was only 0.034.

Chapter 7 and 8 were devoted to interaction studies with tenofovir disoproxil fumarate (DF). The study in *chapter 7* has assessed the effect of rifampin on the pharmacokinetics and tolerability of tenofovir DF in healthy volunteers. All subjects were given tenofovir DF at 300 mg once daily from days 1 to 10. From days 11 to 20 the subjects received tenofovir DF at 300 mg combined with rifampin at 600 mg once daily. The multiple-dose pharmacokinetics of tenofovir (day 10 and 20) and rifampin (day 20) were assessed. The drug-related adverse events (AEs) experienced during this study were mostly mild. Point estimates for the mean ratios of tenofovir with rifampin versus tenofovir alone for the area under the concentration-time curve from time zero to 24 h (AUC_{0-24}), the maximum concentration of drug in plasma (C_{max}), and the minimum concentration of drug in plasma (C_{min}) were 0.88, 0.84, and 0.85 respectively. The 90% classical confidence intervals for AUC_{0-24}, C_{max}, C_{max}, and C_{min} were 0.84 to 0.92, 0.78 to 0.90, and 0.80 to 0.91, respectively, thus suggesting pharmacokinetic equivalence. Similarly, coadministration of rifampin and tenofovir DF did not result in changes in the values of the tenofovir pharmacokinetic parameters. For rifampin, the values of the pharmacokinetic parameters found in this study were comparable to those found in the literature, indicating that tenofovir DF has no effect on the pharmacokinetics of rifampin. In conclusion, adaptation of either the rifampin or tenofovir DF dose for the simultaneous treatment of tuberculosis and human immunodeficiency virus (HIV) infection in HIV-infected patients is probably not required.

In *chapter 8* coadministration of tenofovir DF with efavirenz or nevirapine in HIV-infected patients was studied. Data were retrospectively collected from routine TDM plasma samples. Nevirapine, efavirenz, and tenofovir plasma levels, and tenofovir concentration ratios were analyzed. Six different groups were studied; 200 mg NVP twice daily, 400 mg NVP once

daily, 600 mg EFV once daily, all without tenofovir DF (group 1, 2, and 3, respectively), and the same groups combined with tenofovir 300 mg once daily (group 4, 5, and 6, respectively).

No difference in plasma levels for tenofovir, nevirapine, and efavirenz were found for either one of the combinations studied. Addition of tenofovir DF to efavirenz or nevirapine in HIV-infected patients does not influence the plasma levels of nevirapine or efavirenz. Furthermore, nevirapine and efavirenz have no effect on tenofovir plasma levels and tenofovir concentration ratios.

Efavirenz or nevirapine can be coadministered with tenofovir DF in HIV-infected patients without dose modifications

Single-dose nevirapine is used to reduce mother-to-child transmission (MTCT) in resource limited countries. Enthusiasm for the use of nevirapine for MTCT has been tempered by the observation that exposure of pregnant women to single-dose nevirapine is frequently associated with the development of resistance. The data in *chapter 9* demonstrated that the time to the first undetectable nevirapine plasma concentration varied from 10 days to more than 21 days. This information is valuable for designing intervention studies to prevent the development of nevirapine resistance.

In the general discussion (*Part V*) the main findings are discussed and final thoughts on future research are given.

At TDM-services a close collaboration between laboratory and clinic is essential.

Samenvatting

Met dit proefschrift is getracht om een bijdrage te leveren aan de therapeutic drug monitoring (TDM) van de HIV-behandeling en om een brug te slaan tussen laboratorium en kliniek.
In de inleiding (*Deel I*) van dit proefschrift worden de belangrijkste groepen van de antiretrovirale middelen beschreven en de rol van TDM bij de behandeling van HIV. Daarnaast worden de verschillende hoofdstukken in dit proefschrift kort aangestipt.

In *Deel II* worden onderwerpen die gerelateerd zijn aan de chemische analyse van antiretrovirale middelen gepresenteerd. Een vereiste voor TDM is de plasmaspiegel van de antiretrovirale middelen. In *hoofdstuk 1* is een hoge-druk vloeistof chromatografische methode beschreven voor de gelijktijdige bepaling van proteaseremmers en nevirapine in humaan plasma. Deze methode is eenvoudig, accuraat en precies en deze analyse kan gebruikt worden voor farmacokinetisch onderzoek en TDM in HIV-geïnfecteerde patiënten.
In *hoofdstuk 2* is de stabiliteit van proteaseremmers in humaan plasma bij verschillende temperaturen op lange termijn bestudeerd. Na 18 maanden opslag in de diepvries varieerden de gevonden concentraties tussen de 95% en 108% van de initiële concentraties, dus alle proteaseremmers die bestudeerd werden, zijn stabiel bij -20°C en bij -80°C gedurende tenminste 18 maanden. Het is belangrijk te weten dat dit soort monsters stabiel zijn in de vriezer zodat ook na bewaren accurate uitslagen geproduceerd kunnen worden voor farmacokinetisch onderzoek.
In *hoofdstuk 3* worden de vals-positieve resultaten van efavirenz en rifampicine beschreven in een urine drug-screening assay. Artsen, analisten en onderzoekers moeten zich bewust zijn van de mogelijkheid van vals positieve resultaten.

In *Deel III* wordt aandacht besteed aan de kwaliteit van TDM services. Aangezien plasmaconcentraties van antiretrovirale middelen een essentieel uitgangspunt zijn voor TDM, moeten deze concentraties accuraat geanalyseerd worden. In *hoofdstuk 4* worden de resultaten van de derde ronde van het internationaal interlaboratorium kwaliteitscontrole programma beschreven, waarin deelnemende laboratoria gevraagd werden om proteaseremmers en nonnucleoside reverse transcriptase remmers te meten in humaan plasma. Dit programma liet zien dat er grote verschillen tussen de laboratoria bestaan in hun vermogen om antiretrovirale middelen accuraat te kunnen meten. Slechts 3 van de 30 laboratoria rapporteerden al hun metingen binnen de vooraf gestelde grenzen (80% tot120% van de werkelijke waarde) en van alle metingen viel ongeveer 20% buiten de vooraf gestelde grenzen. De lage concentraties waren moeilijker accuraat te meten dan de medium en hoge

concentraties. Er werd navraag gedaan naar mogelijke foutenbronnen en het bleek dat de meeste fouten van technische aard waren. De resultaten van deze studie kunnen belangrijke gevolgen hebben voor TDM en farmacokinetisch onderzoek.
In *hoofdstuk 5* worden de resultaten van een patiëntencasus, die ook deel uitmaakte van de derde ronde van het internationaal interlaboratorium kwaliteitscontrole programma, gepresenteerd. De casus bestond uit verschillende onderwerpen die gerelateerd zijn aan TDM en aan de deelnemers werd gevraagd om hun aanbevelingen te geven. Van de 30 deelnemers aan het programma, stuurden slechts 16 hun aanbevelingen op. De bloedspiegels van de geneesmiddelen werden correct beoordeeld door ongeveer 90% van de deelnemers. Daartegenover stond dat maar de helft van de aanbevelingen correct was. Daarom is het kwaliteitscontrole programma niet alleen een hulpmiddel om laboratoria opmerkzaam te maken op niet eerder ontdekte analytische problemen, maar ook nuttig om te realiseren dat aanbevelingen betreffende dosisaanpassing, veranderen van regime of comedicatie niet gegeven kunnen worden zonder de juiste kennis.

In *Deel IV* van dit proefschrift worden farmacokinetische onderzoeken gepresenteerd. In *hoofdstuk 6* werd de penetratie van lopinavir in seminaal plasma bestudeerd. De mediane ratio (N=14) van de concentratie van lopinavir in seminaal plasma versus de concentratie van lopinavir in bloed plasma was slechts 0.034, wat betekent dat lopinavir slecht penetreert in seminaal plasma.
In *hoofdstuk 7 and 8* zijn onderzoeken naar mogelijke interacties met tenofovir disoproxil fumaraat (DF) het onderwerp. Het onderzoek in *hoofdstuk 7* heeft het effect van rifampicine op de farmacokinetiek en de verdraagzaamheid van tenofovir DF in gezonde vrijwilligers geëvalueerd. Alle personen kregen éénmaal daags 300 mg tenofovir DF mg gedurende dag 1 tot en met dag 10. Gedurende dag 11 tot en met 20 kregen alle personen éénmaal daags 300 mg tenofovir DF gecombineerd met 600 mg rifampicine. De farmacokinetiek van tenofovir (dag 10 en 20) en rifampicine (dag 20) werd vastgesteld. De geneesmiddel gerelateerde bijwerkingen die gedurende het onderzoek werden gemeld waren voornamelijk mild van aard. De geometrisch gemiddelde ratios voor tenofovir met rifampicine versus tenofovir alleen voor de oppervlakte onder de tijd-concentratie curve van 0 tot 24 uur (AUC_{0-24}), de maximale concentratie van drug in plasma (C_{max}), en de minimale concentratie van drug in plasma (C_{min}) waren respectievelijk 0.88, 0.84 en 0.85. De 90% klassieke betrouwbaarheidsintervallen voor AUC_{0-24}, C_{max} en C_{min} waren 0.84 tot 0.92, 0.78 tot 0.90 en 0.80 tot 0.91 respectievelijk, dus er kan farmacokinetische equivalentie gesuggereerd worden. Derhalve kan gesteld worden dat gelijktijdig gebruik van rifampicine en tenofovir DF niet in veranderingen van farmacokinetische parameters van tenofovir resulteerde. Wat betreft rifampicine waren de farmacokinetische parameters die in dit onderzoek werden

gevonden vergelijkbaar met die uit de literatuur. Dit duidt erop dat tenofovir DF geen effect heeft op de farmacokinetiek van rifampicine. Daarom kan geconcludeerd worden dat dosisaanpassing van zowel tenofovir DF als rifampicine voor de gelijktijdige behandeling van tuberculose en HIV waarschijnlijk niet nodig is.

In *hoofdstuk 8* wordt de combinatie van tenofovir DF met efavirenz of nevirapine bij HIV-geïnfecteerde patiënten bestudeerd. Data werden retrospectief verzameld vanuit routine TDM plasmamonsters. Nevirapine, efavirenz en tenofovir plasmaspiegels en tenofovir concentratie-ratios werden geanalyseerd. Er werden zes verschillende groepen bestudeerd: 200 mg NVP tweemaal daags, 400 mg NVP éénmaal daags, 600 mg EFV éénmaal daags, alledrie zonder tenofovir DF (groep 1, 2 en 3) en dezelfde groepen gecombineerd met éénmaal daags 300 mg tenofovir DF (groep 4, 5 en 6).

Er werd geen verschil in plasmaspiegels gevonden voor tenofovir, nevirapine of efavirenz voor geen enkele combinatie die bestudeerd werd. Toevoeging van tenofovir DF aan een efavirenz of nevirapine bevattend regime bij HIV-geïnfecteerde patiënten, heeft geen invloed op de plasmaspiegels van nevirapine of efavirenz. Daarnaast hebben efavirenz of nevirapine geen invloed op de plasmaspiegels of concentratie-ratios van tenofovir.

Efavirenz of nevirapine kunnen gecombineerd worden met tenofovir DF in HIV-geïnfecteerde patiënten, zonder dat dosis aanpassing noodzakelijk is.

Een éénmalige dosering van nevirapine wordt gebruikt om moeder-op-kind transmissie (MTCT) in derdewereld landen te voorkomen. Het enthousiasme voor nevirapine gebruik wordt verminderd doordat er aangetoond is dat blootstelling van zwangere vrouwen aan een éénmalige dosering van nevirapine regelmatig wordt geassocieerd met de ontwikkeling van resistentie. De data in *hoofdstuk 9* laten zien dat de tijd tot de eerste ondetecteerbare nevirapine spiegel na éénmalige dosering varieerde van 10 dagen tot meer dan 21 dagen. Deze informatie is nuttig bij het ontwerpen van interventie-onderzoek om ontwikkeling van nevirapine resistentie te voorkomen.

In de algemene discussie (*Deelt V*) worden de belangrijkste bevindingen bediscussieerd en enkelen gedachten over toekomstig onderzoek worden gegeven.

Bij TDM-services is een nauwe samenwerking tussen laboratorium en kliniek van groot belang.

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Jacqueline Droste, maart 2006

CURRICULUM VITAE

Jacqueline Adriana Henrica Droste werd geboren op 21 december 1962 te Nijmegen.
Na het behalen van het V.W.O. diploma aan het Canisius College te Nijmegen werd in 1981 begonnen aan het H.L.O. richting analytische chemie aan de OLAN te Arnhem. In 1985 werd het diploma behaald.
Aansluitend werd gestart met een baan op het Laboratorium Endocrinologie en Voortplanting (huidige Afdeling Chemische Endocrinologie) aan het Universitair Medisch Centrum St Radboud te Nijmegen. In 1997 is een overstap gemaakt naar de Apotheek/Klinische Farmacie, alwaar in 2001 gestart werd met het promotieonderzoek.
Ze is getrouwd met en vriendin van André Olthaar en moeder van Stijn.

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